University of Alberta

Role of Rac2 in polymorphonuclear leukocyte activation

by

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This thesis is dedicated with love to my parents, my beloved daughters: Yomna, Hadeel and Yasmeen and to my husband Yasser who helped me every step of the way.

Abstract

Optimal activation of neutrophils is crucial to the innate immune response, while inadequate or excessive activation may prove fatal. Neutrophil activation entails cell motility, release of superoxide anion (O_2^-), and degranulation.

Divergent mechanisms of NADPH oxidase assembly and O_2^- release were revealed in activated neutrophils and eosinophils. The discrepancy seen in extracellular O_2^- production between eosinophils and neutrophils was due to preferential intracellular assembly of NADPH oxidase subunits in neutrophils during O_2^- production. While neutrophils assembled NADPH oxidase intracellularly, eosinophils were shown to assemble NADPH oxidase mainly on the plasma membrane and produce O_2^- extracellularly. This discrepancy may be explained by the different roles these cells play in the immune response.

Anaplasma phagocytophilum infection of neutrophils results in survival of the bacteria inside vacuoles in these cells, and they downregulate NAPDH oxidase expression during chronic infection. However, acute *A. phagocytophilum* infection of neutrophils resulted in intracellular assembly of NADPH oxidase, and the ability to produce O_2^- anion was intact. Neutralization of released O_2^- by the bacterium was proposed as an escape mechanism against neutrophil-mediated killing upon interaction with the neutrophil during infection. *A. phagocytophilum* was shown to reside in oxidase-free vacuoles that lacked membrane components of the NADPH oxidase system as another protective step against the bactericidal effect of the oxidase. This provided a better understanding of mechanisms of host evasion by *A. phagocytophilum*.

The role of Rac2 in neutrophil degranulation was investigated in this thesis. Rac2 has already been well investigated for its function in O_2^- production and actin cytoskeleton dynamics. A novel role for Rac2 in the selective release of primary granules was determined. Chemoattractant stimulation was shown to utilize a common pathway including Rac2 and actin cytoskeleton, leading to granule mobilization and release. Other populations of granules were released normally in $Rac2^{-/-}$ neutrophils, indicating different pathways mediating their secretion. Common signaling pathways were shown to be utilized for both degranulation and O_2^- generation downstream of chemoattractant stimulation. Expression of other isoforms of Rho GTPases could not compensate for Rac2 deficiency. In conclusion, Rac2 was found to have an essential role in mediating immune functions in neutrophils.

To explore the clinical application of the knowledge acquired in this project, we tested the effect of Rac2 deficiency in a lung injury model. Lung tissue injury was substantially diminished in *rac2*$^{-/-}$ mice as assessed by inflammatory parameters. These findings indicate that Rac2 deficiency alters a diverse cascade of events that affect inflammatory cell functions, leading to the attenuation of lung injury. Due to its potent effect in preventing tissue destruction and loss of organ function, Rac2-targeted inhibition may prove to be a novel and effective therapeutic strategy in acute inflammatory disorders.

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Table of Contents

CHAPTER 1 1

1.1 INTRODUCTION 1
1.1.1 Discovery of neutrophils 1
1.1.2 Development and differentiation 1
1.1.3 Role of neutrophils in the immune response 2
1.1.4 Rho GTPases and neutrophil function 3

1.2 Chemoattractant-mediated signaling in neutrophils 9

1.3 Neutrophil chemotaxis 12

1.4 Phagocytosis 16

1.5 Release of toxic mediators 19
1.5.1 Degranulation 19
1.5.2 Respiratory burst 26

1.6 Neutrophil-mediated lung inflammation 36

1.7 STUDY OBJECTIVES AND HYPOTHESES 39

1.8 References 42

CHAPTER 2 60

Divergence of mechanisms regulating respiratory burst in blood and sputum eosinophils and neutrophils from atopic subjects 60

Abstract 61

Introduction 62

Materials & Methods 64

Results 70

Discussion 84

References 91

CHAPTER 3 97

Anaplasma phagocytophilum utilizes multiple host evasion mechanisms to thwart NADPH oxidase-mediated killing during neutrophil infection 97

Abstract 98

Introduction 99

Materials and Methods 102

Results 107

Discussion 130

Discussion 130

References 136

CHAPTER 4 **140**

Rac2 is critical for neutrophil primary granule exocytosis 140

Abstract 141

Introduction 142

Methods 145

Results 150

Discussion 166

References 171

CHAPTER 5 **175**

Neutrophil primary granule release and maximal superoxide generation depend on Rac2 in a common signaling pathway 175

Abstract 176

Introduction 177

Methods 180

Results 184

Discussion 196

References 201

CHAPTER 6 **203**

Preliminary data 203

Rac2 deficiency protects against immune complex-mediated acute lung injury 203

Abstract 204

Introduction 205

Materials & Methods 207

Results 212

Discussion 220

References 224

CHAPTER 7 **227**

7.1 Discussion 227

7.2 Pattern of reactive oxygen species (ROS) production in neutrophils and eosinophils 230

7.3 Mechanism of host evasion by Anaplasma phagocytophilum in neutrophils 232

7.4 Mechanisms regulating neutrophil degranulation in response to chemoattractant stimulation 234

7.5 Rac2 is central in neutrophil mediated signaling 238

7.6 Neutrophils in lung diseases: friend or foe? 241

7.7 Biological significance and clinical implication of targeting Rac2 242

7.8 Conclusions 244

7.9 References 245

List of Tables

Table 1.1.	Classification of neutrophil granule contents.	25
Table 1.2.	Neutrophil-mediated inflammatory disorders.	38
Table 2.1.	Measurement of MFI in eosinophils and neutrophils.	74

List of Figures

Chapter 1

Figure 1.1. Rac2 involvement in diverse neutrophil functions upon f-Met-Leu-Phe stimulation. 11

Figure 1.2. Chemotaxis in neutrophils. 15

Figure 1.3. Steps of phagocytosis in neutrophils. 18

Figure 1.4. Steps of neutrophil degranulation. 24

Figure 1.5. NADPH oxidase in resting human neutrophils. 32

Figure 1.6. The effect of priming on NADPH oxidase in human neutrophils. 33

Figure 1.7. NADPH oxidase assembly and ROS production in human neutrophils. 34

Figure 1.8. ROS production in neutrophils. 35

Chapter 2

Figure 2.1. O_2^- generation in eosinophils and neutrophils. 71

Figure 2.2. Sensitivity of O_2^- generation to Toxin B incubation in eosinophils. 75

Figure 2.3. Expression of Rho GTPases in eosinophils. 77

Figure 2.4. Subcellular fractionation of eosinophils and immunoblot analysis of NADPH oxidase components. 79

Figure 2.5. Confocal analysis of respiratory burst in eosinophils and neutrophils. 81

Figure 2.6. Confocal and deconvolution restoration microscopic analysis of eosinophils and neutrophils in sputum samples. 83

Chapter 3

Figure 3.1. *A. phagocytophilum* binding and invasion of neutrophils. 109

Figure 3.2. *A. phagocytophilum* infected neutrophils exhibit reduced ROS production. 112

Figure 3.3. Induction of neutrophil reactive oxygen species production by *A.phagocytophilum*. 114

Figure 3.4. Neutrophil re-stimulation assay. 117

Figure 3.5. *A. phagocytophilum* interaction results in increased cytochrome b_{558} association with the neutrophil plasma membrane and increased host-cell size. 120

Figure 3.6. Confocal analysis of Rac and $p22^{phox}$ co-localization in uninfected and *A. phagocytophilum*-infected neutrophils. 123

Figure 3.7. Z-sections of *A. phagocytophilum*-infected cells. 124

Figure 3.8. *A. phagocytophilum* detoxifies O_2^- produced in a cell-free system. 127

Figure 3.9. Neither $gp91^{phox}$ nor $p22^{phox}$ co-localize to the *A. phagocytophilum* vacuolar membrane. 129

Chapter 4

Figure 4.1. Absence of primary granule release in chemoattractant-stimulated $Rac2^{-/-}$ neutrophils. 153

Figure 4.2. $CD63^+$ granule translocation is inhibited in $Rac2^{-/-}$ neutrophils. 156

Figure 4.3. Similar granularity in WT and $Rac2^{-/-}$ neutrophils. 157

Figure 4.4. Normal granular morphology in neutrophils from *rac2*$^{-/-}$ mice. 158

Figure 4.5. Priming of $Rac2^{-/-}$ neutrophils fails to reverse the degranulation defect. 161

Figure 4.6. Secondary granule LTF release is equivalent in WT and $Rac2^{-/-}$ neutrophils. 163

Figure 4.7. Release of tertiary granule MMP-9 is similar in WT and $Rac2^{-/-}$ neutrophils. 165

Chapter 5

Figure 5.1. Absence of Rac2 mRNA expression in Rac2 KO BMN. 186

Figure 5.2. Rac2 KO neutrophils express Rac1 and Cdc42. 187

Figure 5.3. NE release is abolished in Rac2 KO BMN. 188

Figure 5.4. Rac2-deficient BMNs fail to secrete MPO in response to DHCB/fMLP. 191

Figure 5.5. Preincubation with SOD did not alter MPO activity in supernatants. 192

Figure 5.6. Maximal $O_2^{\cdot-}$ generation is mediated by Rac2. 193

Figure 5.7. Secondary granule LTF release is equivalent in WT and Rac2 KO neutrophils in response to PMA. 195

Chapter 6

Figure 6.1. Total and differential BALF cell counts. 214

Figure 6.2. Lung histology of control and ALI. 215

Figure 6.3. Reduced vascular permeability in *rac2*$^{-/-}$ mice with ALI. 216

Figure 6.4. Decreased MPO activity in *rac2*$^{-/-}$ mice with ALI. 218

Figure 6.5. Secondary and tertiary granule products in BALF. 219

Chapter 7

Figure 7.1. Proposed signaling pathway mediating superoxide production and degranulation in neutrophils upon f-Met-Leu-Phe stimulation. 240

List of abbreviations

ALI	acute lung injury
ARDS	acute respiratory distress syndrome
Arp2/3	actin-related protein 2/3 complex
CB	cytochalasin B
Cdc42	cell division cycle protein 42
CGD	chronic granulomatous disease
DAG	diacylglycerol
FAD	flavin adenine dinucleotide
F-actin	filamentous actin
f-Met-Leu-Phe	*formyl*-methionyl-leucyl-phenylalanine
GTP	guanosine 5'-triphosphate
G-actin	globular actin
GAP	guanosine 5'-triphosphatase-activating protein
GEF	guanine nucleotide exchange factor
GTPase	guanosine 5'-triphosphatase
ICAM-1	intercellular adhesion molecule 1
IgG	immunoglobulin G
IL-1B	interleukin 1B
IL-8	interleukin 8
IP_3	inositol 1,4,5-trisphosphate
LPS	lipopolysaccharide
LTB_4	leukotriene B_4

LTF	lactoferrin
MAPK	mitogen-activated protein kinase
MPO	myeloperoxidase
MMP-9	matrix metalloproteinase-9
NADPH	nicotinamide adenine dinucleotide phosphate
NE	neutrophil elastase
NO	nitric oxide
NOX	NADPH oxidase
NSF	*N*-ethylmaleimide-sensitive factor
$O_2^{\cdot-}$	superoxide anion
$^{\cdot}OH$	hydroxyl radical
PE	peritoneal exudate
PI3K	phosphatidylinositol 3-kinase
phox	phagocytic oxidase
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocyte
$PI(4,5)P_2$	phosphatidylinositol 4,5-bisphosphate
$PI(3,4,5)P_3$	phosphatidylinositol 3,4,5-phosphate
PX	*phox* homolog
Rac	*ras*-related C3 botulinum toxin substrate
Rho	*ras* homology protein

RhoGDI	Rho guanine nucleotide dissociation inhibitor
ROS	reactive oxygen species
SNAP	soluble NSF attachment protein
SNAP-23	synaptosome-associated protein of 23 kDa
SNARE	SNAP receptor
SOD	superoxide dismutase
TNF	tumor necrosis factor
VAMP	vesicle-associated membrane protein
WASP	Wiskott-Aldrich syndrome protein

Chapter 1

1.1 INTRODUCTION

1.1.1 Discovery of neutrophils

The Russian embryologist, Ilya Metchnikov, first described neutrophils as motile mesodermal cells capable of performing phagocytosis of opsonized particles. He shared a Nobel Prize in Physiology or Medicine in 1908 with Paul Ehrlich of Germany for their discovery of neutrophils and other granulocytes. Paul Ehrlich described the staining properties of granules in white blood cells, and assigned the term "neutrophil" to cells with granules that stain with neutral dyes. Neutrophils are also termed polymorphonuclear leukocytes (PMNs) due to the diverse morphology of their nuclei. They are terminally differentiated white blood cells, produced in the bone marrow by haematopoiesis. They are released into the bloodstream by egress from the bone marrow, where their life span ranges from 6 to10 hours, and are removed from circulation predominantly by Kupffer cells in the liver after undergoing apoptosis [1,2]. Their lifespan increases significantly upon migration into different tissues, where they may survive for up to several days.

1.1.2 Development and differentiation

In a healthy adult, around 10 million neutrophils are released in peripheral circulation every minute; nevertheless, the total number of circulating neutrophils remains constant [3]. The total weight of neutrophils produced during the average human life may exceed the total body weight.

Neutrophil production from granulocyte progenitor cells in the bone marrow follows three main steps: differentiation, proliferation, and cell maturation. In response to granulocyte-colony stimulating factor, granulocyte-macrophage colony stimulating factor and interleukin-3, the colony forming unit-granulocyte/erythroid/macrophage/ megakaryocyte (CFU-GEMM) differentiates into myeloblasts which further differentiate into promyelocytes, myelocytes, band cells and PMN leukocytes. These stages of differentiation are highlighted according to the acquisition of granules, which have characteristic staining properties. Upon maturation, neutrophils are released into the bloodstream.

1.1.3 Role of neutrophils in the immune response

The main contribution of the neutrophil to the innate immune response involves the engulfment and killing of invading bacteria. This is achieved by recognition of factors released during bacterial infection, whether they are bacterial cell wall components or factors generated by inflammatory cells resident in the affected tissue. These factors attract inflammatory cells, mainly neutrophils, to the site of infection and an inflammatory process takes place. In cases of inflammation, neutrophils are the first to arrive at the affected site, where they reach a peak influx at 4 to 6 hours [4]. Following recruitment to sites of inflammation, neutrophils exert immune functions such as phagocytosis, degranulation and the production of cytokines and reactive oxygen species (ROS). Lipid mediators are also produced by neutrophils upon stimulation, where neutrophil chemoattraction is facilitated by the lipoxygenase pathway products leukotriene B_4 (LTB_4) as well as platelet-activating factor (PAF). These multiple factors

contribute to the killing and degradation of invading organisms as well as further recruitment of other cellular populations and propagation of the inflammatory response.

Neutropenia, or low neutrophil numbers, may cause immune deficiency. Individuals who suffer from defects in neutrophil numbers or functions possess a higher predisposition to fatal bacterial and fungal infection [5-10]. Hence, the contribution of the neutrophil to the innate immune response is a cornerstone of human survival, and this is achieved by the diverse capabilities of the neutrophil to attack and inactivate microbes.

1.1.4 Rho GTPases and neutrophil function

Small GTP-binding proteins are monomeric G proteins, with molecular weights ranging from 20 to 25 kDa. The superfamily of small monomeric G proteins contains over 100 members, and is further divided into five subfamilies: Ras, Rho, Rab, Sar1/Arf and Ran families. Small G proteins are involved in cell cycle progression, transcriptional signaling, cell motility and cytoskeletal organization. In order to perform their functions, small G proteins are activated through binding to GTP, a reaction that is catalyzed by guanine exchange factors (GEFs). Guanosine 5'- triphosphatase (GTPase)-activating proteins (GAPs) stimulate GTPase activity and lead to hydrolysis of the G protein-bound GTP to GDP, which causes inactivation of the small G proteins. Monomeric GTPases are thought to act as molecular switches due to their ability to interchange between active and inactive states. There have been many studies establishing the involvement of small GTP-binding proteins in leukocyte function mediating the innate immune response. In neutrophils, bactericidal activities must be tightly controlled to achieve the desirable effect of microbial killing without causing inflammatory damage to the affected tissue(s). Rho GTPases are shown to be important regulators of these immune functions in

neutrophils [11]. Rho GTPases are a subfamily of the vast superfamily of *ras*-related GTPases that are involved in a variety of cellular functions. The members of the subfamily of Rho GTPases include Rho (*ras* homology protein), Cdc42 (cell division cycle protein 42, a homolog of a yeast protein), and Rac (*ras*-related C3 botulinum toxin substrate). Each of these members has a number of isoforms associated with them as well, including Rac, which is present as Rac1, Rac2 or Rac3. Rac1 is expressed ubiquitously, while Rac2 and Rac3 main expression is mainly in the hematopoietic tissues and brain respectively [12]

G proteins have been shown to have an essential role in influencing regulated exocytosis in permeabilized mast cells [13]. Crosslinking of IgE receptors led to the release of histamine and serotonin from mast cell cytoplasmic granules, which was dependent on Rho, Rac and Cdc42 [14]. On the other hand, sustained activation of Rab3a is shown to cause fusion with the plasma membrane and exocytosis using patch clamp analysis of mast cells [15]. The introduction of recombinant G proteins in permeabilized rat mast cells is one approach used to study the effect of these proteins on degranulation.

1.1.4.1 Rac2 and neutrophils

Rac1, Rac2, Rac3, and RhoG are four members belonging to the Rac-subfamily of Rho GTPases. Different genes encode Rac isoforms, and different expression patterns are evident in mouse and humans tissues. Rac1 is expressed ubiquitously, while Rac2 and Rac3 expression is mainly in hematopoietic tissues and brain respectively [12]. Rac1 and Rac2 share 92% amino acid homology and differ mainly in the C-terminus region. Rac2 is the primary isoform in human neutrophils (98%), while Rac1 constitutes the remainder of total Rac isoforms in these cells [16].

Rac2 has been implicated in receptor signaling in neutrophils, where f-Met-Leu-Phe and PAF stimulation lead to its activation and stimulation of Rac2-dependent downstream effectors [17,18]. FcγR binding to its ligand induces Rac activation and pseudopodia formation in phagocytes [19].

Upon Rac2 activation, it dissociates from GDI and binds to GTP through the effect of specific GEFs [20]. In some instances, Vav1 was shown to mediate this exchange [21,22]. In mouse neutrophils, however, P-Rex is the main Rac2 activator in response to chemoattractant stimulation [23]. GAPs are another group of protein that influences the levels of Rac-GTP in the cell through augmentation of GTPase activity. Finally, in resting conditions, RhoGDI prevents the nucleotide exchange and maintains Rac2 in the inactive state (GDP-bound) [24-27].

Lamellipodia and ruffle formation are characteristic appearances of the cell membrane upon stimulation of Rac GTPases [28]. Rac1 activation is essential for actin polymerization in most motile cells [29]. Rac1 has been shown to induce the assembly of stress fibers [30] and membrane ruffles [28] upon microinjection of recombinant proteins in Swiss 3T3 cells. A specific observation suggesting the pivotal role of Rac2 signaling pathways in controlling the actin cytoskeleton is the description of a human mutation of Rac2 where an immunodeficiency syndrome was indicated [31,32]. The main characteristic of this syndrome was the failure of chemotaxis in neutrophils. Another interesting observation was the diminished motility of neutrophils that was accompanied by lower Rac2 expression at the protein and mRNA levels in patients with a type of severe congenital neutropenia [33].

In Rac2 knockout mice, neutrophils have a diminished ability to migrate to tissues. However, chemotaxis was not abolished due to the substitution of Rac1[34]. The expression of constitutively active Rac1 induced F-actin polymerization and augmented cell spreading in NIH 3T3 fibroblasts, which indicates its central role in cell motility [35]. Upon chemoattractant stimulation with f-Met-Leu-Phe and LTB_4, PI3K was shown to mediate Rac2 activation [36,37] in cell-free systems and in human neutrophils [38]. Vav and Sos1 have been implicated in the activation of Rac2 by PI3K [39-41]. Scar/WAVE, a member of the Wiskott-Aldrich syndrome protein (WASP) family, lies downstream of Rac2 and binds to Arp2/3 and profilin to mediate the formation of barbed (fast-growing) ends of F-actin [42-44]. Another mechanism for Rac2 that involves the facilitation of the formation of the barbed end of F-actin is through dissociation of the capping protein gelsolin, which usually binds and prevents elongation of barbed ends in resting cells [45].

Rac2 is a crucial component of the NADPH oxidase system responsible for superoxide production from neutrophils, eosinophils ad macrophages [46-48]. It binds to $p67^{phox}$ upon activation and translocation to the membrane [49]. The Rac2 effector region (residues 26-45) binds to $p67^{phox}$ via the C terminus, which was shown to be essential for $O_2^{\cdot-}$ production. The insert region of Rac2 (residues 124-135) may have a role in cytochrome b_{558} binding [50-52]. Furthermore, Rac2 deficiency profoundly affected $O_2^{\cdot-}$ production in mouse neutrophils [34,53].

In degranulation experiments, exogenously added recombinant Rac1 and Rho were shown to enhance secretion from permeabilized mast cells. Addition of constitutively active Rho and Rac1 increased the proportion of mast cells degranulating in response to GTPγS, a slowly hydrolysable analogue of GTP, and increased $[Ca^{2+}]_i$ [54].

The effects of Rho and Rac on degranulation may be a consequence of their function in cytoskeletal reorganization.

On the other hand, Cdc42 and Rac were implicated in stimulation of secretory granule release from mast cells by a Ca^{2+}-dependent mechanism through inositol trisphosphate (IP_3) formation upon antigen stimulation (cross-linking of IgE receptors) [55].

1.1.4.2 Effects of human dominant negative Rac2 and mouse Rac2 knock out mutations

An interesting observation was made when a male infant was investigated for multiple unresolved infections around the umbilical stump and in the gluteal area [31]. Further investigation of the patient's leukocytes revealed neutrophilia (increase in the number of circulating blood neutrophils), loss of neutrophil chemotaxis, and a mild decrease in ability to kill *Staphylococcus aureus* compared with normal neutrophils. In addition, primary granule release was absent upon f-Met-Leu-Phe or PMA stimulation. However, secondary granule exocytosis, evaluated by LTF release, was normal, which suggested a selective effect of Rac2 deficiency on primary granule exocytosis [31,56]. The infant was found to have a dominant negative Rac2 mutation that lead to the arrest of binding of Rac2 GDP to GEFs and prevented WT Rac2 from being activated. In Rac2 knockout mice, where Rac2 gene expression was absent, bone marrow neutrophils showed a partial defect in superoxide production, reduced chemotaxis, and diminished phosphorylation of p38 and p42/44 MAP kinases [34].

Roberts *et al.* published a study delineating the role of Rac2 in neutrophil function. They produced a *rac2*$^{-/-}$ mouse model generated by targeted disruption of the rac2 gene, which manifested a form of immune deficiency. Neutrophils failed to reach maximal $O_2^{\cdot-}$ production in response to PMA, which was partially corrected by priming

with TNF [34]. Defects in cellular exudate formation, cell spreading and neutrophil chemotaxis were also evident, even after incubation of neutrophils with chemoattractants for twice as long as usual to compensate for any reduction of speed of movement. These effects were suggested to be due to the effects of Rac2 deficiency on the actin cytoskeleton. Filamentous actin formation was measured and *rac2*$^{-/-}$ neutrophils and showed reduced immediate reaction to chemoattractants, which was partially corrected when cells were primed with TNF and granulocyte-macrophage colony-stimulating factor (GM-CSF). Phosphorylation of MAP kinases (p38, and p42/44 MAPK), involved in transcription activation, was diminished in response to f-Met-Leu-Phe stimulation. Altogether these defects led to an immunodeficiency status and a greater susceptibility for infection, although these mice were kept in a specific pathogen-free environment where they ingested autoclaved food and water. An *in vivo* model of fungal (*Aspergillus fumigatus*) infection was utilized to assess for the efficiency of immune response in those mice. This infection resulted in higher mortality and severe systemic infection in the *rac2*$^{-/-}$ mice. These data indicated that two major functions of neutrophils were deficient due to the loss of Rac2 function, these being superoxide production and chemotaxis. However, Rac1 was able to substitute for Rac2 in supporting superoxide production and partial chemotaxis, as Rac1 highly homologous to Rac2 and is expressed at equivalent levels in murine neutrophils [34], in contrast to human neutrophils which preferentially express and utilize Rac2 instead of Rac1.

These data suggest that the immune deficiency seen in *rac2*$^{-/-}$ mice might be caused by a fundamental defect in neutrophil functions, where potential rescue by Rac1 for the Rac2 deficiency is not present. Degranulation responses were another major

function yet to be assessed in *rac2*$^{-/-}$ neutrophils. We obtained these *rac2*$^{-/-}$ mice for the purpose of determining neutrophil degranulation responses in our laboratory.

1.2 Chemoattractant-mediated signaling in neutrophils

Chemoattractant stimulation of neutrophils leads to their migration through a very shallow gradient toward the source of stimulation, ROS production and degranulation (Figure 1.1) [57]. Many products have been shown to have chemotactic effects on neutrophils including formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe), C5a (a product of the complement cascade), LTB_4, and interleukin-8 (IL-8) [58]. Upon binding to specific G-protein coupled receptor on the cell surface, signaling pathways are stimulated leading to cytoskeletal changes that initiate cell movement. The formyl receptor (fMLF-R) is a heterotrimeric G-protein-coupled, pertussis-toxin-sensitive receptor. Neutrophils express two isoforms of fMLF-R: high affinity (FPR) and low affinity forms (FPRL1) [38,59-61]. Upon receptor binding to bacterial (f-Met-Leu-Phe) and mitochondrial proteins [61], the coupled G protein becomes activated and binds to GTP in place of GDP, which leads to the release of α subunit from βγ subunits of the heterotrimeric G protein [62]. This in turn leads to the activation of effector molecules downstream of the α subunit. Downstream effector for G protein coupled receptor can be divided into α subunit effectors that include phospholipase C (PLC) [63], and βγ subunit effectors including: PI3-kinase [64] and PLCβ3 > PLCβ2 > PLCβ1 [63].

Activation of fMLF-R by f-Met-Leu-Phe leads to the stimulation of PLCβ2 and -β3 [65,66], which leads to $O_2^{\cdot-}$ production. Furthermore, phosphoinositide (PI)- specific PLC cleaves $PI(4,5)P_2$ to produce inositol-1,4,5-trisphosphate (IP_3) and DAG. IP_3 leads to intracellular calcium mobilization, whereas DAG activates PKC among other effectors.

f-Met-Leu-Phe-induced neutrophil chemotaxis is thought to be mediated by the βγ subunit through their effect on PI-3 kinase signaling [67,68]. However, actin polymerization in chemoattractant-stimulated neutrophils is partially affected by blocking PI-3 kinase activity using wortmannin [69,70]. It was shown by Traynor-Kaplan *et al* that neutrophils respond to chemoattractant gradient through the production of $PI(3,4,5)P_3$ internal gradient inside the cell, $PI(3,4,5)P_3$ in turn stimulates actin cytoskeleton changes [71,72]. This process was shown to depend on Rho GTPases (Rac, Rho, and Cdc42) [73].

Defective chemotaxis was observed in *rac2*$^{-/-}$ which is comparable to chemotaxis defects seen in the PI-3 kinase γ knockout mice [66,74,75]. However, f-Met-Leu-Phe ability to activate Rac was preserved in PI3K γ knockout mice, which indicates that another molecule substituted for PI3K deficiency and activated GEFs with resulting Rac stimulation.

f-Met-Leu-Phe stimulates Rac-dependent actin assembly [76] through different effectors that include Arp2/3 [77]. Another mechanism for Rac2 effect on filamentous actin formation is the removal of capping protein gelsolin from the end of the actin filament to allow further polymerization [45] (Figure 7.2). Other downstream molecules of Rac2 include partner of Rac1 (POR1) and p-21 activated kinase (PAK), which are required for activation of actin cytoskeleton remodeling [78,79]. These are also strong candidates for Rac2-mediated granule mobilization and exocytosis because the granules were not mobilized to the cell membrane in Rac2$^{-/-}$ neutrophils.

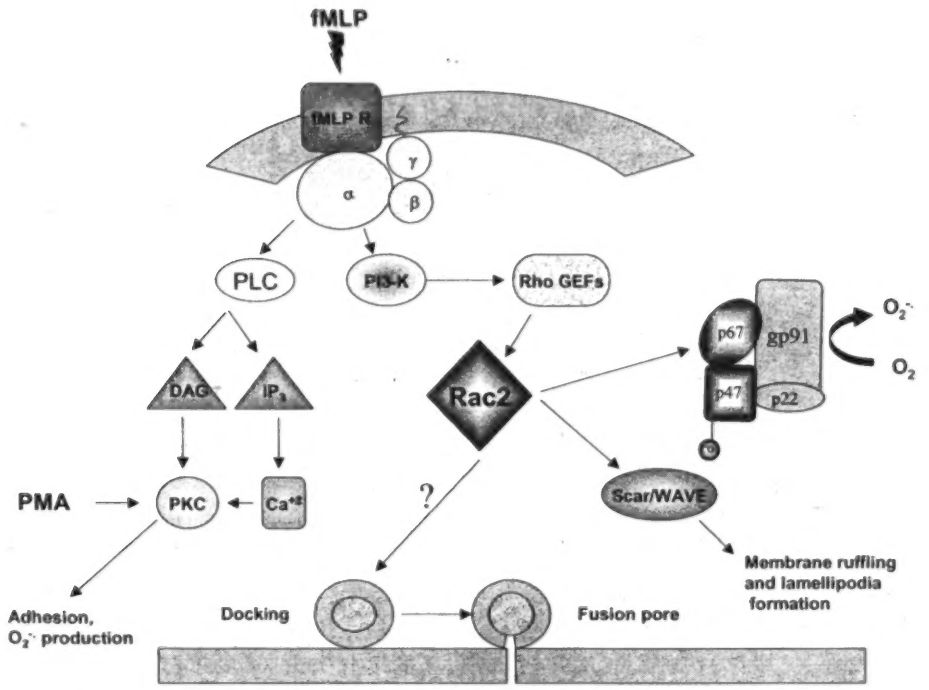

Figure 1.1. Rac2 involvement in diverse neutrophil functions upon f-Met-Leu-Phe stimulation.
Receptor activation leads to activation of PI3K, which promotes Rac2 stimulation through GEFs, causing granule mobilization and release of stored mediators. WAVE is postulated to mediate Rac2 effects on the actin cytoskeleton, which results in the formation of membrane ruffles. Superoxide production occurs upon Rac2 translocation to the membrane and binding to $p67^{phox}$.

1.3 Neutrophil chemotaxis

Circulating neutrophils can detect signs of inflammation, infection and tissue destruction that include chemoattractants such as LTB_4, the bacterial product f-Met-Leu-Phe or the cytokines interleukin 8 (IL-8) and tumor necrosis factor (TNF). Another sign of inflammation that may be detected by neutrophils is upregulation and conformational changes of adhesion molecules on endothelial cells that line the vascular walls. Upon detection of these signals, neutrophils adhere to endothelial cells, and this marks the beginning of the process of transmigration followed by chemotaxis.

Several interactions occur between neutrophils and the endothelial lining of the postcapillary venules and capillaries (Figure 1.2). Binding of selectins (P-and E-selectins), which are expressed on the endothelium, to sialyl $Lewis^x$ on neutrophils leads to an alternating adhesion and release of this bond that results in a rolling motion of neutrophils. Afterwards, neutrophil β_2 integrins (CD11a/CD18 and CD11b/CD18) bind their receptors on the endothelium ICAM-1 (intercellular adhesion molecule) and VCAM-1 (vascular cell adhesion molecule) [80] which results in the firm adhesion of neutrophils to the endothelium. Diapedesis or emigration to the extracellular matrix occurs in response to an increasing gradient of soluble chemotaxins. The directional movement of neutrophils in response to soluble stimuli is hence termed chemotaxis. Chemotaxis is different from chemokinesis, in that the former results from a change in the rate of directional movement in response to chemical substances in the extracellular matrix, while the latter results from a change in random movement of the cell.

Neutrophil kinesis is caused by changes in actin cytoskeleton dynamics [81]. Two types of actin have been shown to alternate in the moving cell: filamentous β-actin (F-

actin) that is formed of polymerized globular β-actin (G-actin) [82]. The formation of F-actin occurs in equilibrium with G-actin to facilitate a more rigid cell content (gel formation with F-actin) or a more fluid cell content (sol formation with G-actin) as required for cell movement. The crucial role of actin polymerization in cell kinesis is established in many studies where cell movement was inhibited using cytochalasins that bind to actin filaments and prevent further addition of actin monomers [83-86]. Furthermore, a heterozygous mutation in the β-actin gene was shown to cause neutrophil dysfunction in humans [87].

The regulation of actin cytoskeleton remodeling is presently under intense investigation. Among many different actin cytoskeletal regulatory pathways identified to date, Rho guanosine 5'-triphosphatases (GTPases) have been shown to be essential regulators of cell motility in leukocytes. During chemotaxis, chemoattractant binding of G-protein-coupled receptors leads to downstream activation of phospholipase C-β [63], and phosphatidylinositol 3-kinase (PI3K) [88]. This in turn stimulates the production of phosphatidylinositol 3,4,5-phosphate (PI (3,4,5) P_3) from PI (4,5)P_2. PI(3,4,5)P_3 activates Rac through its effect on specific guanine exchange factors (GEFs). Cdc42 and Rac-dependent actin polymerization were shown to occur secondarily to formyl (f-Met-Leu-Phe) receptor stimulation [76]. While Cdc42 stimulates *de novo* actin nucleation through actin-related protein 2/3 complex (Arp2/3), Rac was shown to induce cell motility via diverse mechanisms that are both Arp2/3 complex–dependent and independent nucleation [89,90]. Activated Rac is the main Rho GTPase that is responsible for the continuity of this cycle through a positive feedback mechanism [91,92]. Cdc42 is responsible for stabilizing the Rac-dependent leading edge of the neutrophil [93,94]. Leukocyte migration was shown

to be inhibited by dominant-negative Rac1 and Rac2 mutations. A discrepancy was seen between Rac1 and Rac2 mutations, where Rac1 deficiency led to a loss of directional sensing to chemotactic gradients in leukocytes, while a failure of migration was the main observation in Rac2 knockout leukocytes [95].

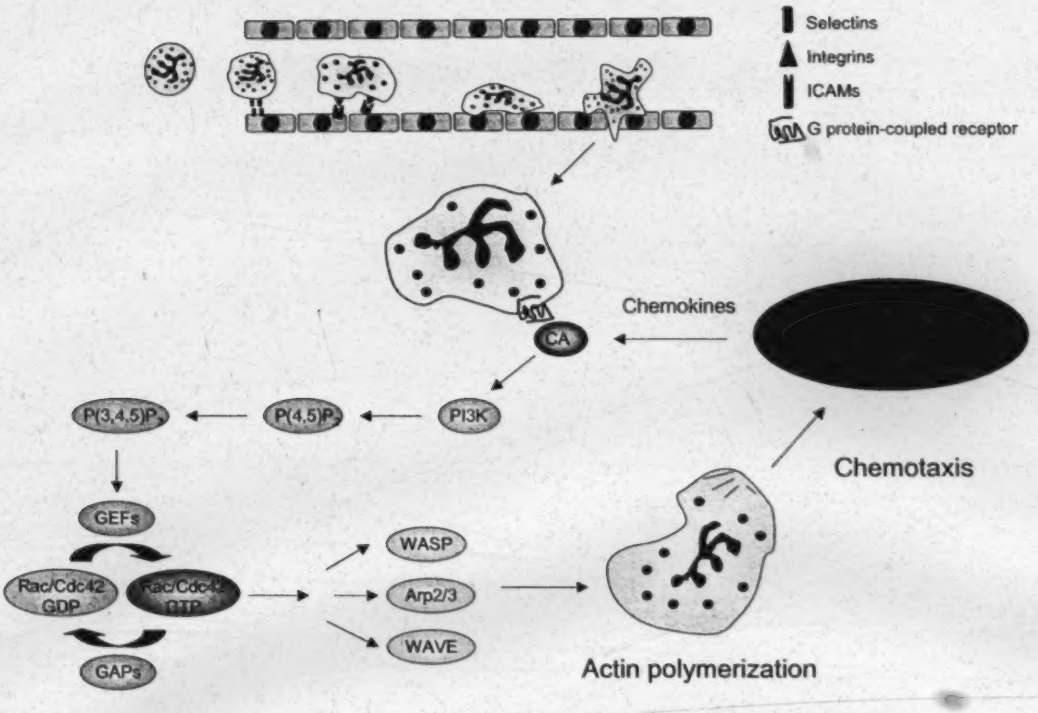

Figure 1.2. Chemotaxis in neutrophils.
Priming of circulating neutrophils leads to receptor mobilization of selectin and integrin, followed by margination and extravasation. Then chemokine binding to G-protein coupled receptors and stimulation of intracellular molecules takes place, leading to actin polymerization in the leading end of the neutrophil to conclude the process by directional movement toward the injury site.

1.4 Phagocytosis

Neutrophils are a pivotal cell population in the innate immune system. The term "professional phagocytes" is classically used to describe neutrophils, macrophages and dendritic cells. However, neutrophil involvement in the immune response was found to encompass phagocytosis but was not necessarily restricted to it.

Phagocytosis is the process of actin-dependent recognition and engulfment of large particles (e.g., bacteria) (Figure 1.3). Phagocytosis is central to host defense mechanisms as well as to tissue repair and embryogenesis. Receptor-mediated recognition of particles is a preparatory step to the engulfment process. Conversely, not all particles are recognized by specific receptors expressed on neutrophils, which was a hindrance in early *in vitro* studies of neutrophil phagocytosis. Opsonization was found to address this problem in which circulating opsonins (e.g. complement factors, antibodies) coat the target and allow receptors to recognize it and begin the engulfment process [96]. Aside from mediating phagocytosis, phagocytic receptor stimulation by opsonized particles also plays a role in delineating the ultimate reaction towards the swallowed particle, and whether the response of the neutrophil is proinflammatory or anti-inflammatory. Engulfed particles are enclosed in a membrane-bound cell organelle termed the phagosome or phagocytic vacuole. Phagosomes are multifunctional organelles that participate in the adaptive and innate immune response through antigen presentation and pathogen killing. In order to achieve these multiple tasks, the phagosome contains various molecules and hydrolytic enzymes that are attained through a maturation process, where the phagosome fuses with lysosomes and granules that constitute up to 40% of vacuolar volume, leading to the formation of phagolysosome [97,98]. In the event that viable

bacteria are engulfed, the bacteria-killing enzymes are assembled at the phagosomal membrane or inserted into the phagosome by intracellular granule fusion. ROS are released into the phagosome to prevent pathogen replication and participate in pathogen killing through its direct oxidative effect and through activation of proteolytic enzymes derived from fused lysosomes. This goal can be reached by alterations in the phagolysosome pH and the influx of hypertonic K^+ which mediates the release of neutrophil elastase (NE) and cathepsin G from proteolgycans heparin and chondroitin sulphate [99,100].

Phagocytosis of immunoglobulin G (IgG) and complement-opsonized particles was shown to entail different members of the Rho GTPases, such as Rac, Cdc42 and RhoA. Cdc42 was shown to enhance membrane projections surrounding IgG-opsonized particles, and Rac mediated its internalization in a process termed Type I phagocytosis [101-104]. In Type II phagocytosis, engulfment of complement-opsonized particles occurred via the complement receptor CR3. Particles were internalized upon contacting actin-lined invaginations in the cell membrane, and this was mediated by RhoA [104]. A deficiency in Rac2 is accompanied by defective phagocytosis in macrophages, which implies its important role in this process [105].

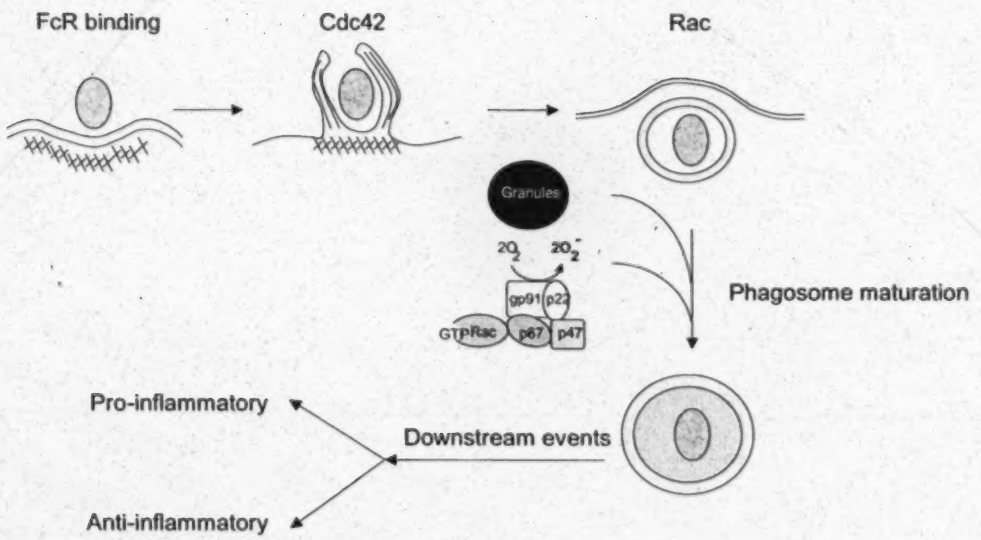

Figure 1.3. Steps of phagocytosis in neutrophils.
Rho GTPases are activated to induce phagocytosis and superoxide release, where Cdc42 enhances filopodial extension and Rac mediates internalization and superoxide generation. Phagosome maturation occurs as a result of granule fusion, and the assembly of the NADPH oxidase system at the phagosomal membrane leads to ROS production. The outcome of phagocytosis can be either pro-or anti-inflammatory according to the nature of engulfed particle

1.5 Release of toxic mediators

1.5.1 Degranulation

In an activated neutrophil, cytoplasmic granules fuse with the phagocytic vacuole following phagocytosis of bacteria, and participate in the intracellular killing machinery. Another possible pathway for granule trafficking is translocation to the plasma membrane where they dock and fuse, leading to extracellular release of granule content, a process termed exocytosis. Granule exocytosis from neutrophils contributes to tissue damage and propagation of the inflammatory response.

Four granule populations are found in the cytoplasm of neutrophils: they are primary (azurophilic), secondary (specific), tertiary granules, and secretory vesicles. Primary granules (peroxidase positive) are the earliest population to appear in the cytoplasm in the promyelocytic stage of neutrophil development. Their production from rough endoplasmic reticulum and Golgi apparatus is arrested in the myelocytic stage when secondary (peroxidase negative) granule formation commences in the Golgi complex. The myelocytic stage is the last mitotic stage in neutrophil life cycle, and is followed by the metamyelocytic stage where tertiary granules and secretory vesicles develop. Neutrophil granules are enveloped by a phospholipid bilayer membrane and consist of matrix, which contains proteins. The granule contents overlap between different granule populations, although there are several proteins which are exclusively expressed in individual granule types. Primary (azurophilic) granules contain many bactericidal proteins and enzymes as myeloperoxidase (MPO), bactericidal/permeability increasing protein (BPI), defensins, NE, and membrane-bound CD63 [106]. MPO, released from neutrophils during activation, is responsible for the production of hypochlorous

acid, a potent antimicrobial agent, from its reaction with hydrogen peroxide and chloride ions. BPI targets Gram-negative bacteria and acts synergistically with defensins, which render target cell membranes more permeable [107,108]. NE has a role in the destruction of the outer membrane in Gram-negative bacteria [109]. Secondary granules contain lactoferrin (LTF), cathelicidins [108] and membrane-bound CD66b [106]. LTF has a high binding affinity for iron atoms and possesses bacteriostatic properties for its ability to deplete iron from the microenvironment. Although the exact role of LTF in host defense remains unknown, it was postulated in many studies that it has an anti-bacterial, anti-fungal, and anti-neoplastic role [110-114]. Cathelicidins have been shown to contribute in bacterial phospholipid hydrolysis [115]. Tertiary granules contain matrix metalloproteinase-9 (MMP-9, gelatinase B), a calcium-requiring enzyme with a high affinity for tissue collagen that is required for neutrophil migration through the basement membrane of blood vessels [116]. Secretory vesicles appear in mature neutrophils and contain plasma proteins (serum albumin) and alkaline phosphatase (AP), as well as CD35 on their membranes [106], which suggests an endocytic origin for these granules (Table 1.1).

Being an essential event in host defense, degranulation has been the focus of many studies aimed at delineating a better understanding of the exact mechanisms controlling this process. The release of granule contents and translocation of granule surface proteins to the plasma membrane are the two explicit results of granule exocytosis. However, there are three basic events that occur during degranulation. First, trafficking of the granule to the target membrane (phagocytic vacuole or plasma membrane), second, granule docking on the target membrane, and third, fusion of the granule membrane with the target membrane, which involves the formation of a fusion

pore followed by pore expansion and release of granule contents. An extra step of vesicle priming has been identified in chromaffin cells where a docked vesicle is transformed into an exocytosis-fit vesicle, this reaction is mediated by SNARE (soluble NSF attachment protein receptor) proteins [117-120].

Sequential granule release in response to a gradient of increasing intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) is evident in neutrophils, where secretory vesicles are the first to be released, followed by tertiary granules, then secondary granules, and finally primary granules. The slower that the granule population is released upon activation, the higher the $[Ca^{2+}]_i$ required [121] for its release.

Ca^{2+} binding proteins in exocytosis

There are a number of Ca^{2+}-binding proteins that have the ability to bind to phospholipids and to the cell membrane that may play a role in regulating granule translocation during exocytosis, such as annexins. The variability in granule interactions with annexins may also be linked to the diverse granule response thresholds to $[Ca^{2+}]_i$ [122,123]. Furthermore, annexin III and I were recruited to the granular membrane upon neutrophil activation, which suggest their role in modulating membrane fusion. Annexin I may enhance secretion in the presence of high Ca^{2+}, while annexin III does not require high calcium for enhanced secretion [124]. Annexin V was shown to be involved in limiting secretion even at very low $[Ca^{2+}]_i$ [124]. These data suggest that annexins I, III, V are involved in controlling the sensitivity of neutrophil secretion to different $[Ca^{2+}]_i$ transients.

Neutrophils degranulate in response to receptor stimulation by agonists termed secretagogues. Secretagogues have differential effects on their ability to release

individual granule populations. Primary and secondary granule contents were shown to be released in the presence of cytochalasin B (CB) and a relevant secretagogue *in vitro* [121]. However, upon receptor stimulation in the absence of CB, secretion involves mainly secretory vesicles and tertiary granules. Thus, cytochalasin B acts as a priming agent for primary and secondary granule release.

According to the SNARE hypothesis (soluble *N*-ethylmaleimide-sensitive fusion [NSF] factor attachment protein receptor), docking and fusion of cytoplasmic granules to the plasma membrane is mediated by a unique interaction of vesicle SNAREs (v-SNAREs) with their corresponding SNARE molecules located on the target plasma membrane (t-SNAREs). Neutrophils were shown to express, v-SNARE, VAMP-2 and SNAP-23, and t-SNARE, SNAP-23, -25 and syntaxin 4 and 6. Syntaxin 4 and SNAP-25 are expressed on the plasma membrane, whereas VAMP-2 is localized to tertiary granules and secretory vesicles [125]. SNAP-23 is localized to secondary and tertiary granule membranes in human neutrophils as well, where it may act as a v-SNARE. In one study, upon neutrophil activation, secondary granule-associated SNAP-23 translocated to the plasma membrane and interacted as a v-SNARE with syntaxin-6, leading to secondary and tertiary granule release [126].

Other secretagogues include f-Met-Leu-Phe, which is a bacterial tripeptide commonly used to study the effects of chemoattractants on neutrophils. Binding of f-Met-Leu-Phe to its receptor (the formyl peptide receptor, which is a G-protein coupled receptor) leads to activation of a heterotrimeric G protein of the $G_{i/o}$ family [127] followed by a rapid activation of tyrosine kinase pathways [128,129] such as MAPK pathway (mitogen-activated protein kinase) [130,131], as well as adaptor proteins [129] and small G

proteins [132]. *Src*-related tyrosine kinase members were shown to mediate exocytosis of secondary granules [133]. Furthermore, translocation of Hck (a *src*-related tyrosine kinase) occurs from primary granules to phagosomes during phagocytosis of serum-opsonized zymosan, and suggests a possible role of this molecule in intracellular primary granule release [134]. Hck and Fgr (*src*-related tyrosine kinases) were found to regulate adhesion-dependent neutrophil degranulation [133]. Moreover, formation of β-arrestin-Hck complexes upon chemokine receptor CXCR1 stimulation, leads to Hck activation and translocation to granule-rich fractions [135]. Thus β-arrestin may contribute to the regulation of chemokine-induced degranulation. Activation of p38 MAP kinase occurs by *src* family kinase-dependent mechanisms and leads to stimulation of primary and secondary granule release [127]. However, downstream molecules of p38 MAP kinase that may be involved in granule exocytosis remain unknown (Figure 1.4).

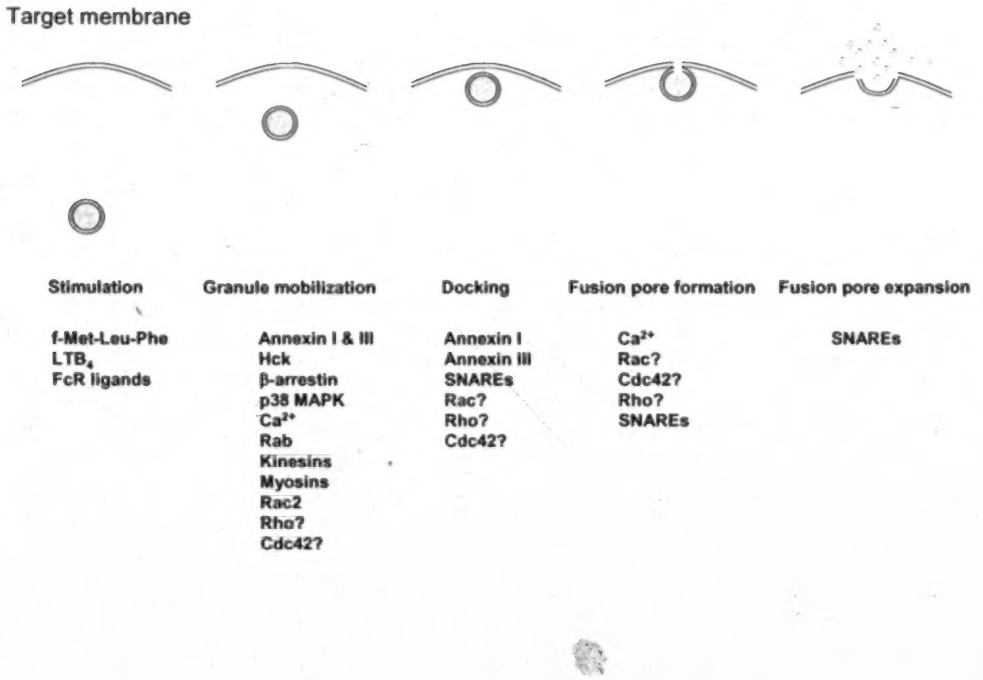

Figure 1.4. Steps of neutrophil degranulation.
Receptor stimulation leads to granule mobilization, which is mediated by a Ca^{2+} gradient, followed by docking of the outer granular membrane to the inner plasma membrane. Shown beneath the stages of granule docking and fusion are the signalling molecules postulated to be involved in each event.

Granules	Azurophilic (primary)	Secondary (specific)	Tertiary	Secretory vesicles
Membrane proteins	**CD63** CD68	CD 11b CD15 CD18 CD66a **CD66b** G-protein α subunit Cytochrome b_{558} NB1 antigen Rap 1&2 TNF-R μPA-R Thrombospondin-R Vitronectin-R Laminin-R fMLP-R Fibronectin-R	CD11b CD18 **CD66b** fMLP-R Cytochrome b_{558} Calnexin ADAM-8 Stomatin LAMP-2 Laminin-R IgE FCR	CD10 CD11b CD13 CD16 CD35 (CR1) CD45 **Alkaline phosphatase** Cytochrome b_{558} Decay-accelerating factor (DAF) fMLP-R μPA-R
Matrix proteins:				
•Proteinases	**Elastase** Cathepsin G Proteinase 3	Collagenase Gelatinase	**Gelatinase**	
•Microbicidal enzymes	**Myeloperoxidase** Lysozyme Azurocidin Neuraminidase (sialidase)	Lysozyme Neuraminidase	Myeloperoxidase Lysozyme	
•Acid hydrolyases	N-acetyl-β-Glucosaminidase Cathepsin B,Cathepsin D β-Galactosidase β-Glucuronidase α-Mannosidase			
•Inhibitors	α-1-antitrypsin Heparin binding protein	**Lactoferrin** PKC inhibitor Heparinase Histaminase		
•Other	Defensins BPI Acid mucopolysaccharide Ubiquitin		Acetyltransferase	Plasma proteins Tetranectin Pro-μPA/ μ PA

Table 1.1. Classification of neutrophil granule contents.
Marker proteins are **bolded**. (*Modified from Neutrophil granules and secretory vesicles. Faurschou, M. &Borregaard, N. Microbes. Infect. 5(14) 1317-27. Copyright 2003 with permission from Elsevier)*

1.5.2 Respiratory burst

Phagocytic cells were meticulously observed for changes occurring during the process of particle internalization in an attempt to understand the mechanism underlying this process. One of the observations that were documented in the early 20th century was increased oxygen consumption associated with phagocytosis, later termed respiratory burst. Originally it was thought that this burst in cell respiration was to provide enough energy for the actual process of phagocytosis. Based on this notion, Selvaraj *et al.* postulated a killing mechanism mediated by this respiratory burst [136]. The first human disease to be described due to inefficiency of oxygen-mediated microbial killing was reported shortly after the discovery of respiratory burst [137]. Chronic granulomatous disease (CGD) was described as a fatal disease resulting from a lack of respiratory burst in phagocytes, with the resulting compromise of microbial killing and uncontrolled bacterial and fungal infections.

NADPH oxidase system

The NADPH oxidase system is comprised of at least six components: flavocytochrome b_{558}, p67^{phox}, p47^{phox}, Rap-1A, p40^{phox} and p21rac (*phox*: phagocytic oxidase). These components are divided into membrane-bound and cytoplasmic proteins.

Flavocytochrome b_{558} is the main membrane-bound component of NADPH oxidase system. Most of the cytochrome b_{558} in the neutrophil (85%) is located within granular membrane during rest, and upon activation, it translocates to the cell membrane or the phagosome [138-143]. Cytochrome b_{558} is a heterodimer of p22^{phox} (α-subunit) and gp91^{phox} (β-subunit). The gp91^{phox} subunit (91 kDa) has two domains that contain the

electron transfer system termed the C-terminus and the N-terminus. The C-terminus is a hydrophilic domain that contains the NADPH and flavin adenine dinucleotide (FAD) binding sites, while the N-terminus is hydrophobic and contains six membrane-spanning α helices [144]. The p22^{phox} subunit is a 21 kDa protein that binds to p47^{phox} [145,146]. Rap1A (Krev 1) belongs to the Ras superfamily of small GTP-binding protein, and is heavily expressed in neutrophils [147-150]. However, the function of Rap1A in oxidase activation is obscure. It was suggested that Rap1A mediates the final activation step of the NADPH oxidase through interaction with cytochrome b_{558} as shown in differentiated HL60 cells using Rap1A mutants [151], although its function appears to be largely modulatory rather than critical for the function of the oxidase based on cell-free assays.

Although cytochrome b_{558} contains the electron transfer machinery, electron transport only happens upon activation of cytosolic components and the assembly of the whole NADPH oxidase system. The p67^{phox} subunit is considered to be an NADPH oxidase (NOX) activator. p67^{phox} is a 60 kDa protein that binds directly to Rac, cytochrome b_{558}, and p40^{phox} [152]. The NOX organizer, or p47^{phox}, is a 45 kDa cytoplasmic protein that is phosphorylated for translocation to the membrane during activation [152]. Its attachment to p67^{phox} and p22^{phox} stabilizes the p67^{phox}/ cytochrome b_{558} complex [144]. The p40^{phox} subunit is a 40 kDa protein that mediates p67^{phox} transport to the membrane through binding to PIPs with its PX domain (*phox* homolog) [153].

The previously mentioned cytoplasmic components were shown to be unable to activate superoxide production upon binding to cytochrome b_{558}, except in the presence of a GTP-dependent factor such as Rac1 or Rac2. Rac1 and Rac2 are functionally exchangeable in their ability to activate respiratory burst NADPH oxidase complex.[154]

Upon receptor activation, the GEF, P-Rex is recruited to the membrane and activated to exchange the GDP group on Rac with a GTP, leading to its activation and membrane translocation [144]. Another GTP-binding protein that modulates oxidase production in neutrophils is Cdc42, which has a high homology to Rac (70%) [155].

The final assembly of all components leads to the production of ROS and superoxide through a succession of events that is almost simultaneous with phagocytosis [97]. In neutrophils, ROS production depends on the nature of stimulus, and is preferentially produced in the phagocytic vacuole rather than outside of the cell to fulfill the neutrophil's function as a classic phagocyte. However, if there is an overwhelming physiological activation of FcγR by opsonized particles, vacuolar closure becomes unattainable and ROS production occurs extracellularly in neutrophils [156,157].

In resting neutrophils, membrane components of NADPH oxidase are situated on the specific granules and secretory vesicle membrane, as well as the insoluble part of the actin cytoskeleton [158,159]. Cytosolic components are present in either monomers (e.g. $p47^{phox}$), heterodimers, or heterotrimers (Figure 1.5) [160-163].

Priming of neutrophils does not lead to NADPH oxidase activation, but rather augments superoxide generation ($O_2^{\cdot-}$) upon stimulation with an additional agent other than the priming stimulus [164,165]. Many agents prime neutrophils, or more specifically, NADPH oxidase, and neutrophils may achieve a primed state within 3-60 min depending on the type of agent. Priming leads to translocation and partial phosphorylation of the cytosolic components of the oxidase (Figure 1.6) [166-172]. Upon full activation of the neutrophil and the NADPH oxidase system, optimal phosphorylation of $p67^{phox}$, $p47^{phox}$,

and p40^{phox} occurs followed ultimately by membrane colocalization of all the subunits (Figure 1.7).

Many physiological (e.g., f-Met-Leu-Phe) and non-physiological agonists (e.g., phorbol myristate acetate, PMA) have been used to study the mechanism of ROS production in neutrophils and neutrophil-like cell lines. f-Met-Leu-Phe is a soluble tripeptide ligand that stimulates NADPH oxidase, among other neutrophil functions, through a G protein-coupled receptor-mediated mechanism. It induces $O_2^{\cdot-}$ production within a short period of time (5 min) and following stimulation, the cell retains the capacity for restimulation by f-Met-Leu-Phe. However, PMA is the most commonly studied stimulus for NADPH oxidase machinery, and acts through an intracellular protein, protein kinase C (PKC), to cause prolonged generation of $O_2^{\cdot-}$ followed by an inability to further produce the superoxide anion due to the exhaustion of cofactors and substrates [173].

Upon stimulation, $O_2^{\cdot-}$ is produced and transformed into other cytotoxic products, mainly through dismutation to hydrogen peroxide either as a spontaneous chemical reaction or catalytically through superoxide dismutase (SOD). Together with released MPO, H_2O_2 oxidizes chloride ions leading to formation of hypochlorous acid, which is the most potent anti-bacterial ROS produced by the neutrophil. $O_2^{\cdot-}$ and H_2O_2 are not thought to participate efficiently in the bacterial killing machinery, except inside the granules, because they are immediately removed by SOD and catalase, respectively [174]. The hydroxyl radical ($^{\cdot}OH$) results from the combination of $O_2^{\cdot-}$ and H_2O_2, in a reaction called the Haber-Weiss reaction. The Fenton reaction has been observed in homogenized cells where a metal (e.g., iron) is required to produce the highly potent $^{\cdot}OH$ and suggested

a role of lactoferrin of neutrophil secondary granules to augment this reaction and enhance $^{\cdot}OH$ production (Figure 1.8) [175-181].

Neutrophils have been shown to produce nitric oxide (NO) during sepsis. However, it is unknown if NO participates in bacterial killing in the phagosome due to the observation that knocking out inducible NO synthase had no or little effect on microbial killing in neutrophils [182].

Eosinophil ROS production during respiratory burst is up to 10-fold greater than that of neutrophils. It was postulated that higher expression levels of the oxidase components might be the reason for high levels of $O_2^{\cdot-}$ production in eosinophils [183-185]. High levels of ROS production may lead to tissue injury, as seen in asthmatic patients [186,187]. However, the detailed mechanisms of NADPH oxidase distribution and assembly in human eosinophils, compared with neutrophils, are unknown.

A. Phagocytophilum infection in neutrophils

In some instances, phagocytosis results in a rather catastrophic event, which is the infection of the neutrophil by internalized bacteria. This may happen due to the ability of these organisms to intervene with the intracellular killing machinery in neutrophils, using a type of host evasion mechanism. *Anaplasma phagocytophilum* is an intracellular Gram-negative bacterium that is known to escape intracellular killing in neutrophils [188], and even reproduces within these cells. Infection by *A. phagocytophilum* causes human granulocytic anaplasmosis (HGA), one of several types of tick-borne diseases that have recently increased due to resurgence in deer populations in North America, particularly in the northeast. One way of escaping the hostile intra-vacuolar environment in neutrophils is to block the generation of ROS by neutrophils through the superoxide-producing

nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex [189,190]. This may be achieved by downregulation of the individual components of NADPH oxidase [191], detoxification of superoxide [192], or prevention of pH changes and phagolysosomal fusion to the vacuole containing *A. phagocytophilum* [193,194]. The mechanism by which *A. phagocytophilum* prevents its killing by NADPH oxidase is unknown.

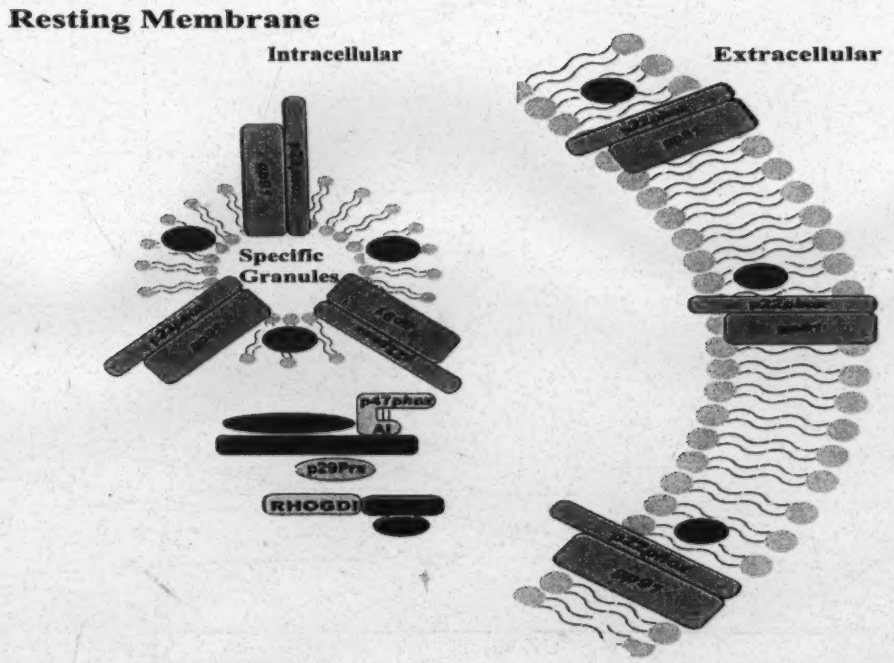

Figure 1.5. NADPH oxidase in resting human neutrophils.
Cytosolic components are present in heterodimeric or heterotrimeric complexes, while Rac2 is present in an inactive from bound to RhoGDI (Rho-GDP dissociation inhibitor) and GDP. Membrane components are present mainly on the granular membrane and to a lesser extent on the cell membrane. (From: *Sheppard FR. et al. J Leukoc Biol. 2005 Nov;78(5):1025-42.*)

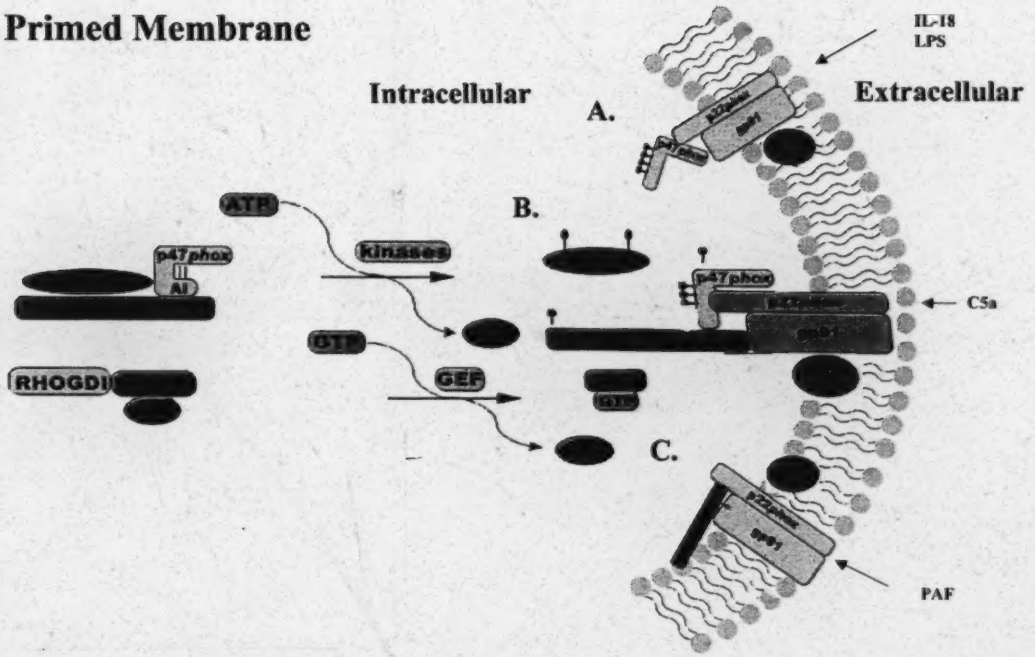

Figure 1.6. The effect of priming on NADPH oxidase in human neutrophils. Selective translocation of cytosolic components to the membrane occurs in response to different stimuli. A) LPS (lipopolysaccharide), IL-18 (interleukin 18) lead to $p47^{phox}$ translocation. B) $p47^{phox}$ and $p67^{phox}$ translocate in response to C5a (complement 5a). C) $p67^{phox}$ translocates in response to PAF (platelet activating factor). (Modified from: *Sheppard FR. et al. J Leukoc Biol. 2005 Nov;78(5):1025-42.*)

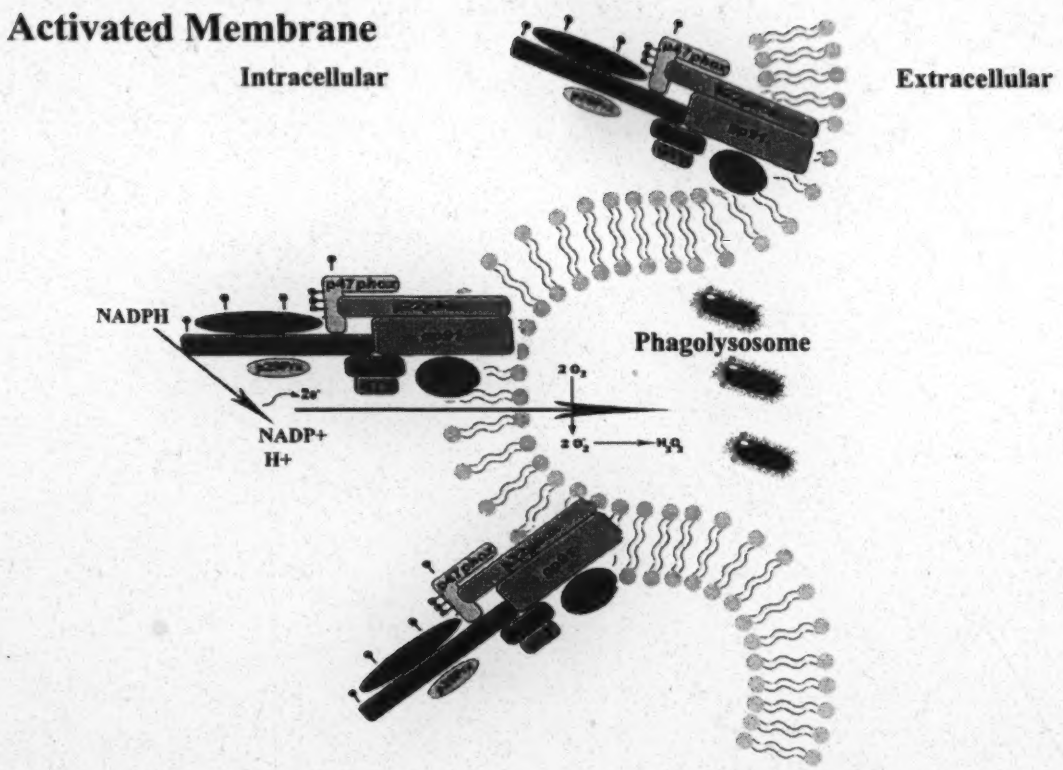

Figure 1.7. NADPH oxidase assembly and ROS production in human neutrophils. NADPH oxidase components are assembled at the membrane leading to electron transfer that is facilitated via the FAD binding domain of $gp91^{phox}$, causing the generation of superoxide anion. (From: *Sheppard FR. et al. J Leukoc Biol. 2005 Nov;78(5):1025-42.*)

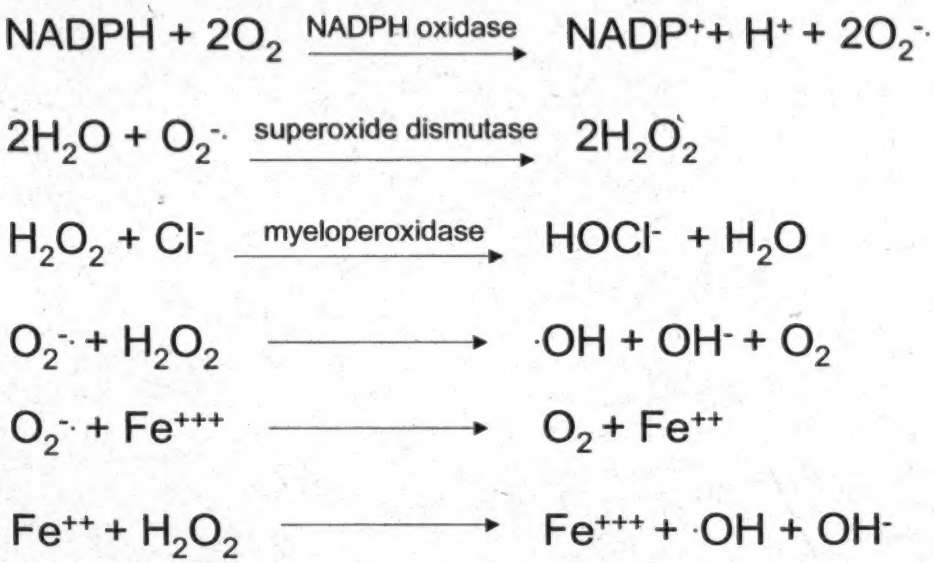
NADPH + 2O2 NADPH oxidase NADP+ + H+ + 2O2-·
2H2O + O2-· superoxide dismutase 2H2O2
H2O2 + Cl- myeloperoxidase HOCl- + H2O
O2-· + H2O2 ·OH + OH- + O2
O2-· + Fe+++ O2 + Fe++
Fe++ + H2O2 Fe+++ + ·OH + OH-

Figure 1.8. ROS production in neutrophils.
NADPH oxidase activation leads to the production of superoxide anion ($O_2^{-\cdot}$), which rapidly dismutates to hydrogen peroxide (H_2O_2) by the effect of superoxide dismutase [195]. This in turn reacts with chloride ion (Cl^-) to produce hypochlorous acid in a reaction catalyzed by myeloperoxidase stored in primary granules. The Haber-Weiss reaction does not occur normally except in the presence of iron, and is termed the Fenton reaction, that occurs through two steps and lead to the production of hydroxyl radical.

1.6 Neutrophil-mediated lung inflammation

In 1970, neutrophils were implicated as a major pathogenic cell in the development of adult respiratory distress syndrome (ARDS) and acute lung injury (ALI) [196,197]. This changed the scientific view of neutrophils from being a beneficial cell in the immune response to a potentially hazardous and injurious to the host under certain circumstances (Table 1.2). Bernard *et al.* published a study where they determined that 30% of severe sepsis patients in the intensive care unit would develop ARDS, with mortality at 28 days of up to 35% [198].

Studying the mechanism of ALI in humans has been daunting due to the diversity of contributing factors and initial illnesses that cause the systemic inflammatory syndrome leading to ALI. High mortality rates have been another factor contributing to the difficulty of studying ALI. However, many studies have reached a solid understanding of the mechanism of ALI in humans through the employment of animal models.

Neutrophils have been shown to play a vital role in inducing tissue destruction seen in ALI [199,200]. Upon initial stimulation by factors leading to injury, alveolar epithelial cells and macrophages release TNF and IL-1B to induce the upregulation of adhesion molecules on vascular endothelium. Activation of adhesion molecules on endothelial cells facilitates neutrophil extravasation into the submucosal tissues and ultimately into the airway lumen, where injury is initiated [4].

Following recruitment to the lungs, neutrophils undergo mediator release that consists of granule-derived products, secreted by degranulation, and ROS. An imbalance

between neutrophil oxidants/proteolytic enzymes and their natural inhibitors in the lungs has been implicated in the pathogenesis of ALI [201,202].

The known functions of Rac2 in neutrophil activation suggests that it may also play a role in mediating neutrophil inflammatory responses. However, the role of Rac2 in regulating neutrophilic inflammation has never been investigated. We chose an immune complex-mediated model of ALI in the mouse, first described in 1978 [203], as an experimental model to specifically determine the contribution of Rac2 to severe inflammatory responses in the lung.

Organ	Disorder
Lung	Adult respiratory distress syndrome
	Asthma
	Asbestosis
	Emphysema
	Idiopathic pulmonary fibrosis
Kidney	Glomerulonephritis
	Interstitial nephritis
Heart	Myocardial reperfusion injury
	Ischemic heart disease
	Rheumatoid arthritis
	Gout
Gastro-intestinal tract	Inflammatory bowel disease
Systemic	Scleroderma
	Vasculitis
Others	Burns
	Frostbite
	Dermatitis
	Malignant neoplasms at sites of chronic inflammation

Table 1.2. Neutrophil-mediated inflammatory disorders.
Uncontrolled activation of neutrophils results in the release of oxidants and proteolytic enzymes, leading to an inflammatory response, and in severe cases, tissue destruction and loss of organ function. (Reprinted from *Immunopharmacology of neutrophils, P.G. Hellewell and T. J. Williams. Chapter 1: the neutrophil, page 3, Copyright (1994), with permission from Elsevier)*

1.7 STUDY OBJECTIVES AND HYPOTHESES

This research project is aimed at the delineation of signaling pathways involving Rac2-mediated activation of neutrophils. The overall hypothesis of the study is that Rac2 is an essential regulatory protein in activation of neutrophil mediator release, and that Rac2 plays an important role in the induction of the inflammatory response caused by neutrophil degranulation and respiratory burst. The thesis is composed of three main objectives:

1) Characterization of NADPH oxidase assembly through Rac2 localization in human neutrophils and eosinophils during stimulation of the respiratory burst.
2) To determine the role of Rac2 in neutrophil degranulation.
3) To determine the effects of Rac2 deficiency in an immune complex-mediated model of acute lung injury.

Characterization of NADPH oxidase assembly in human neutrophils upon stimulation

The respiratory burst is an important event that is implicated in microbial killing and the propagation of the inflammatory response. ROS are released from neutrophils upon assembly of the NADPH oxidase that entails Rac2 protein activation. Rac2 is the most highly expressed Rac subfamily member in human neutrophils. The $p67^{phox}$ subunit binds to Rac2 preferentially during NADPH oxidase activation in neutrophils. It has been postulated that neutrophils do not produce $O_2^{\cdot-}$ in quantities similar to eosinophils [204]. We tested the hypothesis that ROS production sites are different in eosinophils from neutrophils while producing equivalent amounts of total ROS.

Microbial killing by superoxide produced by NADPH oxidase is postulated to be inefficient in *A. phagocytophilum* infection. *A. phagocytophilum* is able to survive inside neutrophils in the host, and this in turn suggests the presence of one or more mechanisms to avoid microbial killing machinery inside the vacuole containing the bacterium. In this thesis, we tested the hypothesis that *A. phagocytophilum* inhibits ROS production through the prevention of NADPH oxidase assembly and $O_2^{\cdot-}$ production.

Investigation of the role of Rac2 in neutrophil granule release employing a rac2 knockout mouse model

Based on earlier studies, Rac2 has been implicated in mast cell and neutrophil degranulation. However, it was not clear if the defect in degranulation was due to a defect in granulogenesis or exocytosis. Hence we employed a *rac2* gene knockout mouse model to assess the involvement of Rac2 in degranulation, and to determine whether a defect may be due to the failure of granule formation, granule mobilization, or fusion with the membrane and exocytosis.

To determine the effect of Rac2 deficiency on immune complex-mediated lung injury

Immune complex-mediated acute lung injury is a unique model for acute lung injury where the main cell type implicated in tissue destruction is the neutrophil [199,205,206] through the production and release of inflammatory cytokines (TNF, IL-1β), ROS, and toxic mediators stored in the primary granules such as myeloperoxidase and elastase [207,208]. I hypothesized that Rac2 deficiency will have desirable effects leading to the attenuation of tissue injury in the lungs of Rac2 knockout mice. Assessment of the injury

was aimed at delineating the numbers of neutrophil in BALF, measurement of neutrophil proteolytic enzymes in BALF, determination of the preservation of tissue architecture, and investigating the degree of loss of vascular integrity.

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Chapter 2

Divergence of mechanisms regulating respiratory burst in blood and sputum eosinophils and neutrophils from atopic subjects

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D. Abdel Latif contribution to this study is as follows:

- Isolation of human neutrophils and eosinophils from peripheral human blood
- PMA stimulation of isolated human granulocytes
- Double labelling, confocal microscopy and image capturing of granulocytes
- Wrote the methods section for cytospin staining and confocal microscopy sections

Abstract

Eosinophil respiratory burst is an important event in asthma and related inflammatory disorders. However, little is known concerning activation of the respiratory burst NADPH oxidase in human eosinophils. Conversely, neutrophils are known to assemble NADPH oxidase in intracellular and plasma membranes. We hypothesized that eosinophils and neutrophils translocate NADPH oxidase to distinct intracellular locations, consistent with their respective functions in O_2^--mediated cytotoxicity. PMA-induced O_2^- release assayed by cytochrome *c* was 3.4-fold higher in atopic human eosinophils than neutrophils, although membrane-permeable DHR-123 showed similar amounts of release. Eosinophil O_2^- release was dependent on Rac, as it was 54% inhibited by *C. difficile* Toxin B (400-800 ng/ml). In eosinophils stimulated with PMA, a pronounced shift of cytosolic Rac to p22*phox*-positive plasma membrane was observed by confocal microscopy, while neutrophils directed Rac2 mainly to intracellular sites co-expressing p22*phox*. Similarly, *ex vivo* sputum eosinophils from asthmatic subjects exhibited predominantly plasma membrane-associated immunoreactivity for Rac, while sputum neutrophils exhibited cytoplasmic Rac2 staining. Thus, activated sputum eosinophils, rather than neutrophils, may contribute significantly to the pathogenesis of asthma by extracellular release of tissue-damaging O_2^-. Our findings suggest that the differential modes of NADPH oxidase assembly in these cells may have important implications for oxidant-mediated tissue injury.

Introduction

Respiratory burst is an important event in many inflammatory conditions, characterized by production of superoxide anion (O_2^-) and related reactive oxygen species (ROS), including H_2O_2 and OH^- (1). Eosinophils from atopic subjects generate enhanced levels of ROS (2,3), which may directly injure tissues in the airways (4) and can react with eosinophil peroxidase to produce further tissue-damaging microbicidal products (1). Neutrophils are also well characterized for their ability to release O_2^- (5). Interestingly, neutrophils from asthmatic subjects have been shown to generate more O_2^- than those from normal individuals (6,7). In all cell types exhibiting respiratory burst, regulated generation of O_2^- is dependent on assembly and activation of the normally latent NADPH oxidase complex in cell membranes (8,9).

The molecular mechanisms associated with NADPH oxidase assembly and activation have been studied in more detail in neutrophils (5,8,10-13) and cell-free assays (10,14) than in eosinophils (15-19). This complex is composed of five essential subunits, the membrane-bound cytochrome b_{558} (a complex of two subunits, p22*phox* and gp91*phox*) which associates with cytosolic subunits Rac, p47*phox*, and p67*phox* during stimulation (8). Rac, a small monomeric GTP-binding protein of approximately 21 kDa, belongs to the Rho family of guanine triphosphatases (GTPases) (20) and is bound to cytosolic Rho guanine dissociation inhibitor (RhoGDI) under basal conditions. Rac1 shares 92% amino acid homology with Rac2, and these are functionally interchangeable in their ability to activate NADPH oxidase (14,21-23), while other GTPases, including K-Ras, Rap1A, Rap1B, RhoA and Cdc42Hs, are unable to activate this complex in cell-free assays (24). Rac2 expression is limited to hematopoietic cells (25). During respiratory

burst, p47*phox* and p67*phox* (in a complex with p40*phox*) become phosphorylated and translocate to cell membranes to bind cytochrome b_{558}. Concurrent phosphorylation of a putative guanine nucleotide exchange factor induces dissociation of Rac from RhoGDI and binding of activated Rac-GTP to gp91*phox* and p67*phox* in the membrane (24).

Studies on eosinophil NADPH oxidase activation have shown strong similarities between eosinophils and neutrophils in assembly and activation of this complex. However, eosinophils generate up to 10-fold more extracellular O_2^- than neutrophils, which may be caused by elevated expression of NADPH oxidase in eosinophils (15-17,26,27). Several reports have alluded to the possibility that stimulated neutrophils mainly generate O_2^- intracellularly to assist in phagocytic killing (28,29). However, there is a lack of direct evidence of expression and location of NADPH oxidase in human eosinophil versus neutrophil respiratory burst.

In this study, we hypothesized that eosinophils preferentially assemble NADPH oxidase in the plasma membrane in order to generate extracellular O_2^-. In addition, we investigated whether human eosinophils express Rho-related GTPases, which are required for O_2^- release through NADPH oxidase activation. Using a combination of subcellular fractionation and confocal laser scanning microscopic techniques, we describe a clear divergence in the spatial distribution of the critical oxidase regulator Rac between eosinophils and neutrophils both *in vitro* and *ex vivo*. Understanding the molecular details regulating this process and distinct patterns of regulated O_2^- release in these two cell types may contribute to development of novel and cell-specific therapeutic targets to modulate mediator secretion in asthmatic inflammation.

Materials & Methods

Materials. Nycodenz was purchased from Gibco BRL Life Technologies, Ltd (Grand Island, NY). Baculovirus-generated recombinant Rac2 and rabbit polyclonal antiserum to recombinant human Rac2 were generously provided by Dr. G.M. Bokoch, Scripps Research Institute, La Jolla, CA (30,31). Rabbit polyclonal antibody to RhoGDI was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-Rac antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY) and BD Transduction Labs (Mississauga, ON). Dihydrorhodamine-123 (DHR-123) was obtained from Molecular Probes (Eugene, OR). Rabbit polyclonal antisera anti-recombinant human p47*phox* and p67*phox* were kindly supplied by Dr. William Nauseef, University of Iowa, Iowa City, IA. Mouse monoclonal anti-human p22*phox* and gp91*phox* proteins were generously provided by Dr. Mark T. Quinn, Montana State University, Bozeman, MT. *Clostridium difficile* Toxin B was purchased from List Biological Laboratories (Campbell, CA).

Preparation of eosinophils and neutrophils. To isolate eosinophils, peripheral blood (100 ml) was obtained from mild atopic asthmatic and atopic nonasthmatic subjects displaying eosinophilia >2%, and who were not receiving oral corticosteroids (32,33,33). Briefly, whole blood was subjected erythrocyte sedimentation in 6% dextran, and upper phase cells were centrifuged on a single-step Ficoll gradient before highly purified $CD16^-$ eosinophils (>99%) were isolated by negative immunomagnetic selection. For neutrophils, 50-100 ml of peripheral blood was obtained from normal subjects (except for experiments using DHR-123, in which both eosinophils and neutrophils were obtained from the same atopic donor), which was subjected to erythrocyte sedimentation in 6%

dextran, followed by density centrifugation on Ficoll. Using this isolation technique, neutrophil purity usually averaged >98%.

Measurement of O_2^- release from eosinophils. Generation of extracellular O_2^- from cells in suspension was measured as previously described (19). Briefly, cells (1-2 x 10^6) were suspended in 1 ml microcuvettes containing supplemented phosphate buffered saline (PBS+), pH 7.4 (with 1.2 mM $MgCl_2$, 5 mM KCl, 0.5 mM $CaCl_2$, 5 mM glucose, and 0.1% BSA) and 50 μM ferricytochrome *c* at 25°C. The mixture was blanked at 550 nm in a Beckman DU 640 spectrophotometer (Beckman Instruments Inc, Mississauga, ON) before adding PMA at doses ranging from 1-1000 ng/ml. Superoxide dismutase-inhibitable absorbance was calculated using $\varepsilon = 2.11 \times 10^4\ M^{-1}\ cm^{-1}$ for reduced cytochrome *c*. To inhibit O_2^- production, *C. difficile* Toxin B (40-800 ng/ml) was added to 1 x 10^7 cells/ml in RPMI-1640 (Gibco BRL) containing 15% FCS and incubated at 37°C for 2-20 h before treatment with PMA.

Dihydrorhodamine-123 (DHR-123, 1 μM), a membrane-permeable probe sensitive to oxidation by ROS to produce fluorescent rhodamine-123, was incubated at 37°C for 10 min with cells (1-2 x 10^6) before adding 1 μg/ml PMA for 10 min using a modification of previously published protocols (34,35). Cells were then subjected to flow cytometric analysis (FACScan, Becton Dickinson, San Jose, CA). Values of MFI were obtained from gated regions on dot plots for each sample.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Highly purified batches of eosinophils and neutrophils (≥ 99%) were subjected to total RNA extraction using a QIAGEN RNeasy MiniKit (Mississauga, ON) using 2 x 10^6 cells per extraction (producing 0.2-1 μg RNA) (19). Primer sequences used for detecting Rac1/2 mRNA

were generated in our laboratory for Rac1 (product size = 575 bp), with forward and reverse sequences as follows: (2-24 bp) 5'-TGCAGGCCATCAAGTGTGTGGTG-3' and (554-576 bp) 5'-CAACAGCAGGCATTTTCTCTTCC-3'. Rac2-specific primers (product size = 576 bp) were (3-25 bp) 5'-GCAGGCCATCAAGTGTGTGGTGG-3' and (556-578 bp) 5'-TAGAGGAGGCTGCAGGCGCGCTT-3', respectively. Both sets of primers are intron-spanning (36,37). Reactions were carried out in a PTC 100 Thermal Controller (M-J Research, Inc, Watertown, MA) using an annealing temperature of 57°C (Rac1-specific primers) or 56°C (Rac2-specific primers). Non-reverse-transcribed samples were included as controls.

Western blot analysis. Samples were subjected to acrylamide gel electrophoresis and transferred to Immobilon PVDF membrane blots (19). Primary mouse monoclonal antibodies to Rac (1:1000), p22*phox* (1:1000), gp91*phox* (1:1000), or rabbit polyclonal antibodies to Rac2 (1:500), RhoGDI (1:500), p47*phox* (1:1000), and p67*phox* (1:1000) were used before introduction of secondary antibodies (1:5000 sheep anti-mouse IgG or 1:5000 donkey anti-rabbit IgG conjugated to horseradish peroxidase, Amersham Canada Ltd, Oakville, ON). Chemiluminescence was developed by addition of SuperSignal substrate solution (Pierce, Rockford, IL).

Subcellular fractionation of eosinophils and marker enzyme assays. Eosinophils were subjected to homogenization through a ball-bearing cell homogenizer (HGM Precision Engineering, Heidelberg, Germany), followed by production of postnuclear supernatant from which organelles were separated by linear density gradient centrifugation (19,38).

PMA-stimulated eosinophils ($\geq 5 \times 10^7$ cells) were prewarmed to 37°C in 5 ml PBS+ with 250 U/ml catalase and 50 U/ml superoxide dismutase for 5 min (19). PMA

was added to a final concentration of 500 ng/ml for 8 min at 37°C. The reaction was terminated by addition of 10 ml ice-cold PBS+ and cells were homogenized as described above.

Profiles of marker enzyme activities were obtained using previously reported techniques (19,33,38). Fractions enriched in cytosol (lactate dehydrogenase, LDH), plasma membrane/small secretory vesicles (CD9), crystalloid granules (eosinophil peroxidase), and crystalloid/small granules (β-hexosaminidase) were determined in supernatants and pellets using modifications of microtitre plate assays (33). Plasma membrane activity was determined by dot blot analysis with monoclonal antibody to CD9 (32).

Double labeling and confocal laser scanning microscopy. Granulocyte cytospins (50 µl of 0.8 x 10^6 cells/ml in RPMI-1640 supplemented with 20% FCS) were prepared by Cytospin 2 centrifugation (Shandon Ltd, Astmoor, Runcorn, UK) as previously optimized (39). Primary labeling was carried out with 20 µg/ml mouse monoclonal anti-human Rac, specific for both Rac1 and Rac2 (mouse IgG_{2b}, Upstate Biotechnology, Inc.). Immunoreactivity to Rac was detected using 1.4 µg/ml Rhodamine Red-conjugated goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) (33,40). A blocking step with PE Goat F $(ab)_2$, anti-mouse IgG (H+L), human adsorbed (40µg/ml, Cedarlane Laboratories Limited) was done for 1 hour prior to application of the second primary antibody. Slides were double-labeled with 20 µg/ml anti-human p22*phox*. Bound anti-p22*phox* was detected by incubating 20 µg/ml BODIPY FL-labeled goat anti-mouse IgG (Molecular Probes). Mouse IgG_1 and IgG_{2b} (20 µg/ml) were used as isotype controls (R & D Systems, Sigma). Cells were counterstained with DAPI nuclear stain. Slides were

mounted with 30 µl antibleaching agent (0.4% n-propyl gallate [Sigma] in 3:1 glycerol:TBS) before coverslip attachment, and then examined using a 40X objective on a Zeiss confocal laser scanning microscope (Carl Zeiss Microimaging, Thornwood, NY). Images were collected and processed as described (33).

Sputum collection and analysis. Sputum was induced from normal and well-characterized asthmatic subjects by inhalation of nebulized saline at increasing concentrations (up to 5%) (41). Atopic asthmatic subjects [5], as determined by the skin prick test, exhibited FEV_1 scores of <80% of predicted values at the time of diagnosis. All asthmatics were on medication (inhaled corticosteroids and β-agonists). Following inhalation of nebulized saline, subjects rinsed their mouths, then coughed sputum into sterile collection containers. Mucus plugs were manually removed and fixed immediately in 4% paraformaldehyde in PBS for 2 h. Samples were paraffin-embedded via butanol in a tissue-array fashion (42). For immunohistochemical analysis, samples were sectioned and stained as described above for cytospins for Rac and major basic protein (MBP) immunoreactivity. To detect MBP, 1% mouse monoclonal antibody to human MBP (BMK-13, generated in-house (43)) was applied to sections. Bound BMK-13 was detected by 5 µg/ml BODIPY FL-conjugated goat anti-mouse IgG. Slides were analysed by confocal laser scanning microscopy on a Zeiss system described above, and by deconvolution restoration microscopy using a DeltaVision microscope system (Applied Precision, Issaquah, WA).

Data presentation. Enzyme activities corresponding to granule, membrane and cytosol constituents following fractionation were expressed as frequency distributions (32). Data

were analyzed by ANOVA or Kruskal-Wallis statistical comparison with individual samples compared by Tukey's or Dunn's multiple comparison test.

Results

Extracellular vs. intracellular O_2^- levels generated by eosinophils and neutrophils. Eosinophils and neutrophils generated extracellular O_2^- in response to PMA stimulation, although there was a significant discrepancy in amounts of O_2^- produced from each cell type. Continuous spectrophotometric measurement of cytochrome *c* reduction in response to increasing doses of PMA (Figure 2.1a, b) revealed that the peak rate of O_2^- release was 3.4-fold higher from eosinophils than neutrophils, similar to a value of 3.2-fold obtained previously (44) (Figure 2.1c). At the maximally stimulating dose of 50 ng/ml PMA, the average peak rate of O_2^- release from eosinophils was 15.9 ± 0.6 nmol $O_2^-/10^6$ cells/min compared with 6.2 ± 0.7 nmol $O_2^-/10^6$ cells/min for neutrophils.

These values are close to those obtained for PMA-induced O_2^- production in eosinophils and neutrophils in a previous study, which reported 13.2 ± 1.1 nmol $O_2^-/10^6$ cells/min in eosinophils and 7.2 ± 1.3 nmol $O_2^-/10^6$ cells/min in neutrophils in response to 100 ng/ml PMA (27). In addition, the peak rate of O_2^- generation in eosinophils always exceeded that of neutrophils at all doses of PMA >1 ng/ml (Figure 2.1c). These findings support those of earlier reports (17,27,45). Although the response reached a plateau within 5-10 min of stimulation, the saturation appears not to be due to consumption of total cytochrome *c* in the reaction because lowering the concentration of cells did not result in loss of the plateau (data not shown).

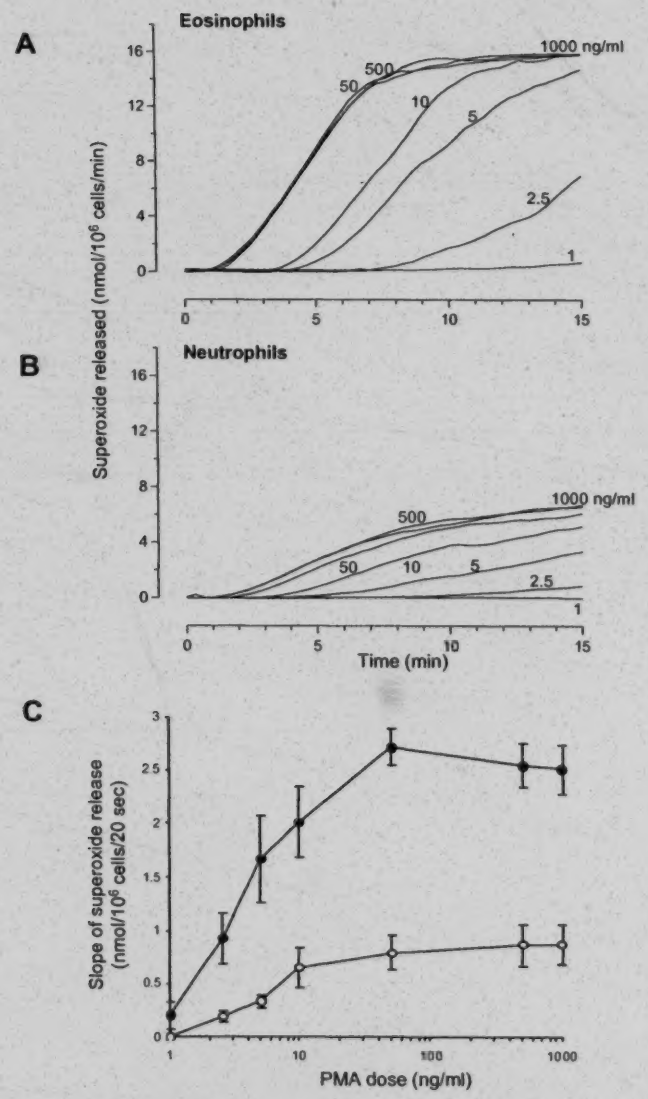

Figure 2.1. O_2^- generation in eosinophils and neutrophils.
(a) Eosinophils (1 x 10^6) stimulated by PMA (1-1000 ng/ml). (b) Neutrophils (2 x 10^6) stimulated under similar conditions. (c) Slopes of O_2^- release in response to increasing doses of PMA in eosinophils (●) and neutrophils (○) in response to increasing doses of PMA over a 15-minute period. Results were averaged from 4-5 separate experiments. (Figure 2.1 was generated by M. Steward)

A fluorescent probe assay coupled with flow cytometric analysis was used to determine ROS production in eosinophils and neutrophils. The membrane permeable fluorochrome DHR-123 is unable to discriminate between intracellular and extracellular production of ROS as it primarily reacts with H_2O_2, which is also membrane-permeable and has the capacity to re-enter cells during PMA incubation prior to analysis on FACS (46). Thus, total ROS production (both intra- and extracellular) may be measured using this fluorescent probe. Cells were incubated in the presence of DHR-123 at 37°C for 10 min prior to addition of 1 μg/ml PMA for 10 min at the same temperature, and then subjected to flow cytometric analysis. Using this assay, we observed an average increase of 3,014 MFI units in PMA-stimulated eosinophils compared 3,857 MFI in similarly stimulated neutrophils (Table I). The increase in MFI in both cell types was significant compared with unstimulated cells ($p < 0.01$ for eosinophils, $p < 0.05$ for neutrophils). However, there was no significant difference in the PMA-stimulated values of MFI between cell types. These data suggest that neutrophils may generate similar total amounts of intra- and extracellular ROS compared with eosinophils during respiratory burst, in contrast to previously published data (17,27,44,45).

Is Rho-related GTPase activation essential for O_2^- release in eosinophils? We determined whether Rho-related GTPases are required for NADPH oxidase activation in eosinophils by incubating cells with *C. difficile* Toxin B. As shown in Figure 2.2, increasing doses of Toxin B reduced O_2^- generation in response to a suboptimal dose of PMA (10 ng/ml). Inhibition was significant at 400 ng/ml Toxin B ($p < 0.05$), and was further suppressed at 800 ng/ml (54% inhibition, $p < 0.01$). No significant inhibition was detected after 2 and 16 h of incubation with Toxin B at these doses (data not shown).

These findings indicate that Rho-related GTP-binding proteins may be involved in regulation of PMA-induced O_2^- release in eosinophils.

	Eosinophils	Neutrophils
Unstimulated	253 ± 56	1,112 ± 148
PMA-stimulated (1 μg/ml)	3,267 ± 905**	4,969 ± 903*

Table 2.1. Measurement of MFI in eosinophils and neutrophils.
Measurement of MFI in eosinophils and neutrophils loaded with DHR-123 (1 μM) and stimulated for 10 min with PMA (1 μg/ml) at 37°C. Values represent average MFI obtained in five separate experiments. * $p < 0.05$, ** $p < 0.01$.

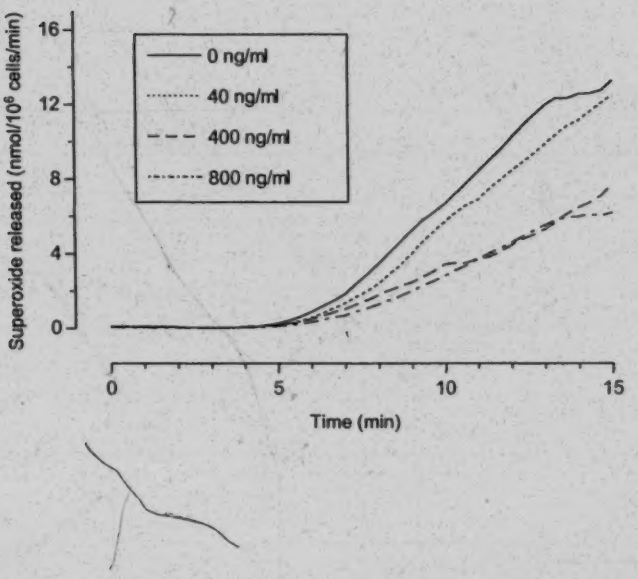

Figure 2.2. Sensitivity of O_2^- generation to Toxin B incubation in eosinophils. Eosinophils (1 x 10^6) were incubated at 37°C in the presence of 0-800 ng/ml Toxin B for 20-24 h and stimulated by 10 ng/ml PMA. These results represent mean ± SEM of responses from three measurements (two different donors). Toxin B decreased maximal PMA-induced O_2^- release by 42% at 400 ng/ml ($p < 0.05$) and by 53% at 800 ng/ml ($p < 0.01$). (Figure 2.2 was generated by M. Steward)

Eosinophil expression of Rac1, Rac2, and Cdc42 message and protein. In order to determine expression of Rho-related proteins in eosinophils, we generated primers detecting human Rac1 (using Genbank Access. No. NM_006908.2) and Rac2 (using GenBank Access. No. M29871). Primers specific for Rac1 and Rac2 generated products migrating to similar sizes as those from human neutrophils (575 bp for Rac1, 576 bp for Rac2) (Figure 2.3a).

We also generated primers for human Cdc42. However, these were not intron-spanning as the gene sequence for human Cdc42 (GenBank Access. No. M35543) does not contain any introns (47). Consequently, we obtained PCR products for Cdc42 in non-reverse transcribed control RNA samples, indicating contamination by genomic DNA (data not shown).

Western blot analysis of whole cell homogenates using antibodies raised against human Rac1, Rac2, and Cdc42 demonstrated that Rac and Cdc42 proteins were expressed in eosinophils (Figure 2.3b). We were unable to determine whether eosinophils express Rac2 in preference to Rac1, since currently available antibodies do not specifically recognize Rac1.

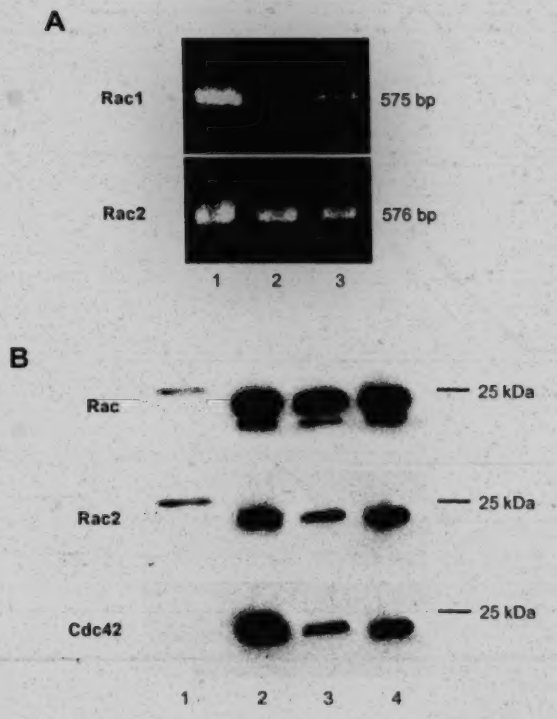

Figure 2.3. Expression of Rho GTPases in eosinophils.
(a) RT-PCR analysis of RNA samples from guinea pig peritoneal eosinophils (lane 1), human eosinophils (lane 2), and human neutrophils (lane 3). RNA samples were treated with DNase before RT-PCR analysis to remove genomic contamination. Results are representative of 5 preparations. (b) Immunoblot analysis of Rac1/2 and Cdc42 expression in eosinophils. Samples loaded were baculovirus-generated recombinant Rac2, 0.1 pg (lane 1), guinea pig macrophages (78%), 20 μg (lane 2), human eosinophils (97%), 20 μg (lane 3), and human neutrophils (99%), 20 μg (lane 4). Antibodies to Rac (Transduction Labs) and Rac2 (Dr. G.M. Bokoch) were used. Baculovirus-generated Rac2 protein migrated at a higher apparent M_r than cell samples due to additional amino acids upstream of the Rac2 sequence. Note that the mouse monoclonal anti-human Rac1 from BD Transduction Labs did not discriminate between Rac1 and Rac2. Similar results were obtained with an antibody to human Rac from Upstate Biotechnology Inc. (results not shown). Results are representative of three separate experiments. (Figure 2.3 was generated by M. Steward and P. Lacy)

Translocation of NADPH oxidase components to cell membrane fractions occurs in parallel with O_2^- generation. Membrane translocation of cytosolic NADPH oxidase components (Rac1/2, p47*phox*, and p67*phox*) has been previously shown to correlate with O_2^- production in neutrophils (30,31) and guinea pig eosinophils (18,19). In order to determine whether similar translocation occurred in human eosinophils, 5×10^7 eosinophils were subjected to subcellular fractionation and immunoblot analysis before and after stimulation with PMA. Figure 2.4 shows the profiles of marker enzyme activities and corresponding immunoreactivities for these components. Positions of intracellular organelles determined by marker enzyme assays before and after PMA stimulation were unchanged (data not shown), indicating that PMA did not alter organelle densities. Before stimulation, Rac, RhoGDI, p47*phox*, and p67*phox* were predominantly expressed in cytosolic fractions, while the cytochrome b_{558} subunits, p22*phox* and gp91*phox*, were localized to plasma membrane-rich fractions. Following 8 min of stimulation with PMA (500 ng/ml), p47*phox* and p67*phox*, but not RhoGDI, translocated to fractions containing cytochrome b_{558} immunoreactivity, although no discernable Rac translocation occurred. A small shift in immunoreactivities for p22*phox* and gp91*phox* towards lower density fractions (fractions 5 and 6) was observed in PMA-stimulated cells, suggesting that cytochrome b_{558} may be present in a population of small secretory vesicles which fuse with the plasma membrane upon activation.

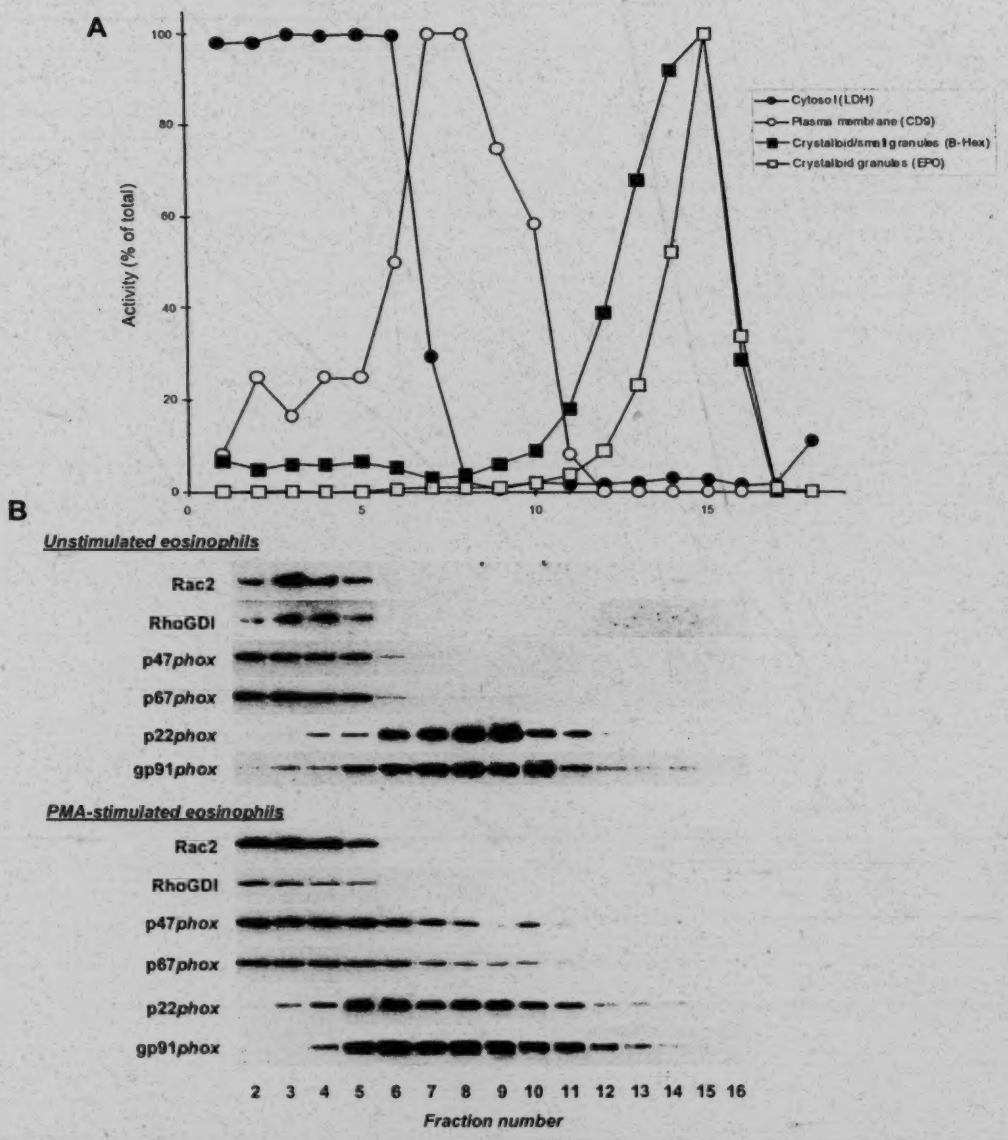

Figure 2.4. Subcellular fractionation of eosinophils and immunoblot analysis of NADPH oxidase components.

(a) Marker enzyme profiles of eosinophils after subcellular fractionation, indicating locations of cytosol (LDH), plasma membrane/small secretory vesicles (CD9), and crystalloid granules (EPO). (b) Immunoblot analysis of Rac2, RhoGDI, p47*phox*, p67*phox*, p22*phox*, and gp91*phox* expression in unstimulated and PMA-stimulated cells. Cells were stimulated with 500 ng/ml PMA for 8 min before termination of reaction. (Figure 2.4 was generated by M. Steward and P. Lacy)

*Double-labeling of Rac and p22*phox *demonstrates divergence in eosinophil and neutrophil NADPH oxidase assembly.* Intracellular sites of NADPH oxidase assembly have not yet been determined in eosinophils, although neutrophils are well known for their expression of oxidase components in specific granules (48,49). We sought to determine whether eosinophils translocate Rac to cell membranes in correlation with extracellular O_2^- generation.

Rac immunofluorescence was cytosolically distributed in unstimulated eosinophils (Figure 2.5a), while p22*phox* was membrane-associated (Figure 2.5b) with some intracellular staining. Overlaid images showed little colocalization between Rac and p22*phox* under basal conditions (Figure 2.5c). Stimulation of O_2^- release in eosinophils was found to result in a pronounced translocation of Rac from the cytoplasm to the cell periphery (Figure 2.5d), to colocalize with membrane p22*phox* immunofluorescence (Figure 2.5f).

Resting neutrophils exhibited a similar pattern of Rac and p22*phox* immunofluorescence to that of eosinophils (Figure 2.5g-i). However, stimulated neutrophils showed a distinct translocation of Rac from that of eosinophils, in which increased intracellular Rac staining was detected which colocalized with the p22*phox* label (Figure 2.5j,l). These findings suggest that, unlike eosinophils, activated NADPH oxidase is predominantly assembled at intracellular sites in neutrophils.

Eosinophils and neutrophils in asthmatic sputum samples exhibit distinct patterns of Rac distribution. We examined eosinophils in sputum from atopic asthmatics to examine patterns of Rac staining *ex vivo*. We were surprised to find that granulocytes in sputum exhibited similar Rac staining to those stimulated *in vitro*.

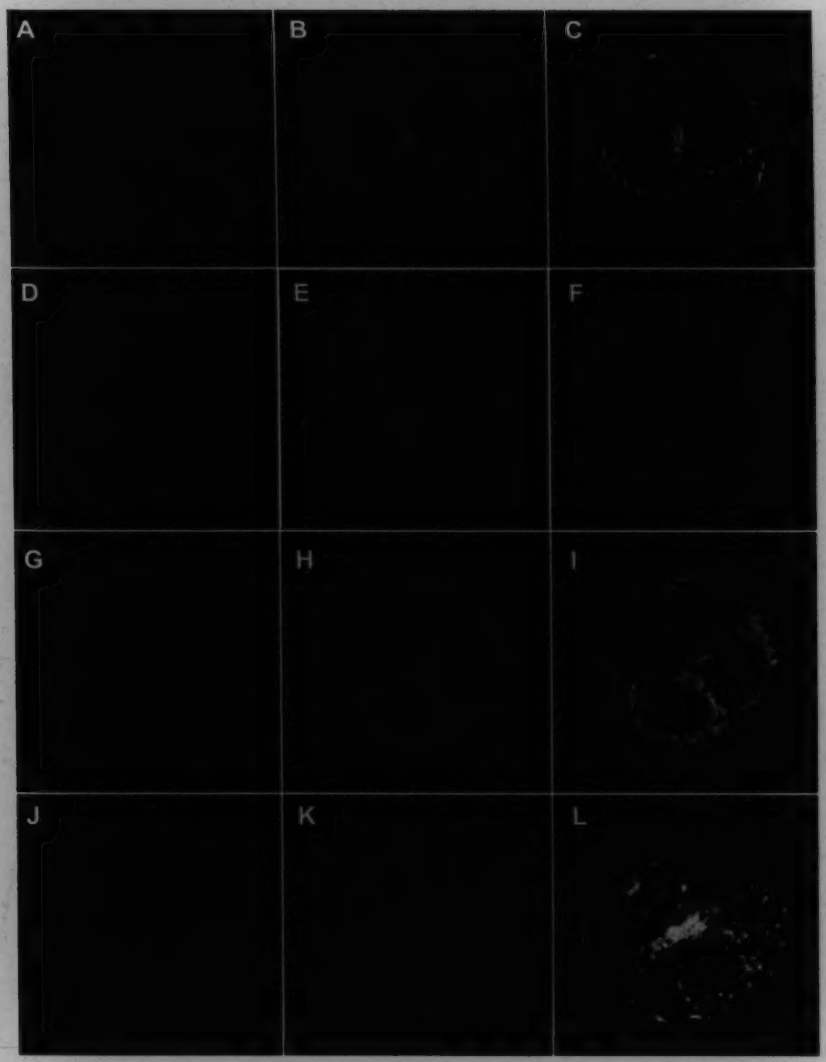

Figure 2.5. Confocal analysis of respiratory burst in eosinophils and neutrophils. Cells were double-labeled for Rac and p22*phox*, followed by DAPI nuclear counterstain (blue). Rac immunofluorescence (a, d, g, j), shown in red, was overlaid with green p22*phox* immunofluorescence (b, e, h, k) to determine colocalization as indicated by yellow-orange color (c, f, i, l). Anti-Rac from Upstate Biotechnology, Inc. was used in these experiments. Panels (a-c) show resting eosinophils, while (d-f) PMA-stimulated (500 ng/ml) eosinophils. Neutrophils, shown in (g-i), were compared with PMA-stimulated cells (j-l). Original magnification 40X. (Figure 2.5 was generated by D. Abdel Latif)

As shown in Figure 2.6b-f, morphologically intact MBP^+ eosinophils in asthmatic sputum samples exhibited peripheral immunostaining for Rac along cell membranes, analogous to that found in PMA-stimulated eosinophils (Figure 2.5d). In contrast, MBP^+ eosinophils in normal sputum samples, which were rare, expressed low levels of cytosolic Rac (Figure 2.6g), comparable to unstimulated peripheral blood eosinophils (Figure 2.5a). Moreover, MBP^- polymorphonuclear neutrophils in asthmatic sputum, identified based on their multilobular nuclear morphology using DAPI nuclear counterstaining, displayed substantial intracellular Rac staining (Figure 2.6h), similar to those stimulated *in vitro* with PMA (Figure 2.5j).

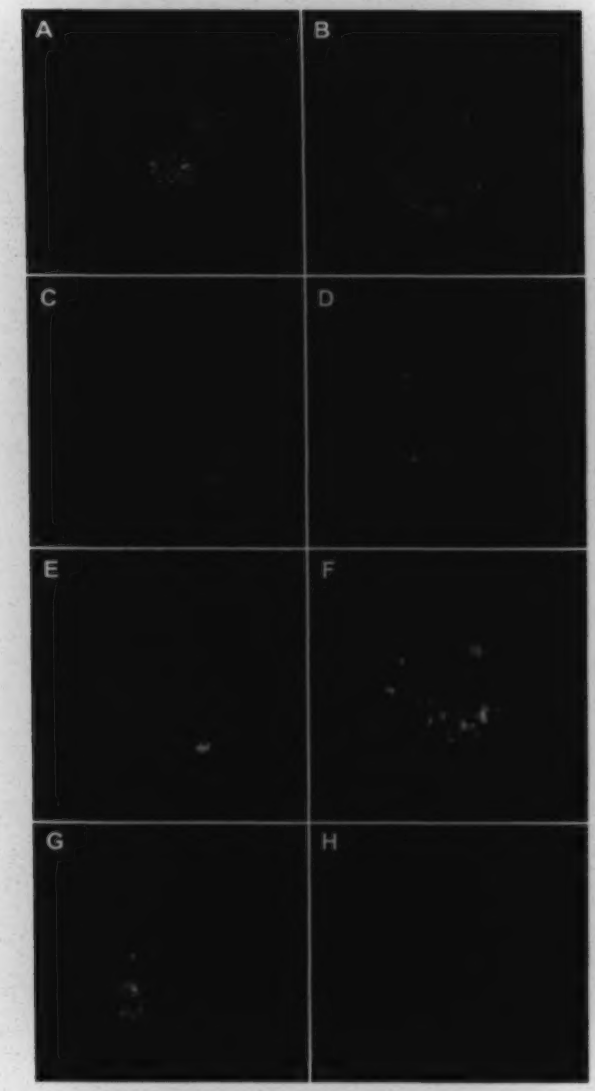

Figure 2.6. Confocal and deconvolution restoration microscopic analysis of eosinophils and neutrophils in sputum samples.

Confocal images are shown for sputum eosinophils from normal (a) and asthmatic (b) subjects following labelling with anti-Rac, shown in red fluorescence, and MBP, as indicated by green. Deconvolution restoration microscopy was also carried out on eosinophils (c-f) and neutrophils (h) from asthmatic sputum. All panels were produced from combined images of double labeling. A sputum eosinophil from a normal donor (g) is shown for comparison. Original magnification 63X. Sections of asthmatic sputum shown here are representative of samples obtained from five atopic asthmatics. (Figure 2.6 was generated by D. Abdel Latif (A, B) and S. Musat-Marcu (C-H))

Discussion

Translocation and assembly of NADPH oxidase is essential for regulated O_2^- generation in phagocytes. Activation of this complex is critically dependent on receptor stimulation of intracellular regulatory Rho-related GTPases, principally Rac1 or its homolog Rac2. In this study, PMA-induced O_2^- generation in eosinophils was shown to require Rac stimulation of NADPH oxidase, as demonstrated by its sensitivity to Toxin B inhibition. Although Toxin B inhibits Rho, Rac and Cdc42 by monoglucosylation at Thre-37 or Thre-35 (50), it is likely that the inhibitory effect of Toxin B on O_2^- production in eosinophils was mediated through blockade of Rac1 or Rac2. This is based on findings from cell-free assays which demonstrated that only Rac1 and Rac2, and not Rho or Cdc42, were able to activate NADPH oxidase in reconstituted lipid bilayers (24).

In addition, human eosinophils were shown to express both Rac1 and Rac2 mRNA, although only Rac2 protein could be positively identified by Western blot analysis. This is similar to guinea pig eosinophils, which may produce both isoforms (18,19). Human neutrophils, in contrast, have been shown to predominantly express Rac2 protein (21,31). Rac2 binds with a 6-fold higher affinity with p67*phox* than Rac1 in two-hybrid assays, suggesting that it may be a more effective inducer of oxidase activity than Rac1 (51). Previous studies have demonstrated expression of p40*phox*, p47*phox*, and p67*phox*, and cytochrome b_{558} in human eosinophils (15,17,52-55), but did not show expression of Rho-related GTPases. Eosinophils may preferentially express Rac2 rather than Rac1 based on a shared hematopoietic lineage with neutrophils, although we were unable to determine Rac1 expression based on our Western blot data.

Translocation of oxidase components during respiratory burst has not previously been demonstrated in human eosinophils. In our study, translocation of cytosolic p47*phox* and p67*phox* to plasma membrane at 8 min of PMA stimulation in eosinophils was similar to earlier observations on guinea pig eosinophils (18,19) and correlated with observations in neutrophils (30). This study demonstrated that p47*phox* and p67*phox* translocated to the membrane during respiratory burst on an equimolar basis with Rac. The majority of cellular cytochrome b_{558} is localized to specific granule membranes in neutrophils, which is transferred to plasma or phagosomal membranes upon activation (48,49,56). Cytosolic p47*phox* and p67*phox* associate with cytochrome b_{558} following phosphorylation of specific serine/threonine sites (8). These bind through SH3 and pleckstrin homology domains to allow association of Rac to plasma membrane and p67*phox*. Collectively these subunits work to initiate electron transfer from NADPH to FAD through cytochrome b_{558}, ultimately resulting in formation of O_2^- from O_2 on the external or lumenal surface of the membrane (8).

The mobilization and assembly of NADPH oxidase to cell membranes in human eosinophils correlated with maximal O_2^- generation as determined by cytochrome *c* reduction assays. This procedure measures only extracellular production of O_2^-, since O_2^- and cytochrome *c* are membrane-impermeable. Previous studies using immunoblot analysis have demonstrated that eosinophils produce express more NADPH oxidase than neutrophils, which was thought to explain why eosinophils generate more O_2^- than neutrophils (15,17,18). However, the discrepancy in O_2^- production between eosinophils and neutrophils may also be attributable to neutrophils preferentially generating O_2^- inside cells. We tested this notion by stimulating cells with PMA in the presence of DHR-

123, which can detect intracellular ROS production by reacting with H_2O_2 and forming a fluorescent product inside the cells (46). Total ROS production was found to be equivalent in PMA-stimulated neutrophils and eosinophils using DHR-123. These findings suggest that neutrophils preferentially generate O_2^- intracellularly, which may partially account for the discrepancy in cytochrome *c* measurements of O_2^- release from eosinophils and neutrophils.

The confocal data in this study supported the possibility that eosinophils generate most of their O_2^- extracellularly in correlation with the translocation of Rac to the cell membrane. We observed by confocal microscopy that, in striking contrast to neutrophils, eosinophils preferentially translocated Rac to the plasma membrane rather than intracellular sites following PMA stimulation. Neutrophils did not appear to translocate Rac2 to plasma membrane, and instead directed Rac2 to intracellular sites in association with p22*phox* of PMA stimulation. Interestingly, eosinophils are able to phagocytose extracellular *E. coli* and *S. aureus* but are unable to kill these as efficiently as neutrophils (57), which was believed to correlate with an inability to modify amino acids through the peroxidase-H_2O_2-Cl^- system. Our findings indicate that eosinophils may be less efficient at killing due to the lack of significant intracellular O_2^- production.

Oxidase assembly may occur in two distinct pools in the neutrophil which are regulated by different pathways. The mechanism of action of PMA is not well understood, although it is frequently assumed to activate NADPH oxidase through protein kinase C. Wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3K), has been shown to block only intracellular production of O_2^- and not extracellular release induced by PMA (58). This indicates that a PI3K-dependent pathway may activate only

intracellular O_2^- production in neutrophils, while a PI3K-independent pathway regulates extracellular release of O_2^-. In contrast, the effect of wortmannin on eosinophil respiratory burst is not well understood. Thus, while eotaxin-induced O_2^- release was inhibited by wortmannin in human eosinophils, it was without effect on LTB_4-induced respiratory burst in guinea pig eosinophils (1). It remains to be determined whether eosinophils utilize a PI3K-independent pathway in PMA-induced oxidase activation to release O_2^- extracellularly.

We did not detect significant expression of oxidase components in eosinophil crystalloid granule-enriched subcellular fractions, but rather in plasma membrane/light membrane fractions (including small secretory vesicles, Figure 2.4), which supports the findings of Calafat and colleagues (53). NADPH oxidase activation is therefore unlikely to involve crystalloid granules in eosinophils. This is in contrast to neutrophils, which express cytochrome b_{558} in specific granules (48,49,53,59). The majority of NADPH oxidase activity in neutrophils undergoing arachidonate- or PMA-induced respiratory burst was found to localize to specific granules (58,60,61) . The function of cytochrome b_{558} expression in neutrophil specific granules is thought to be associated with NADPH oxidase activation following fusion of the specific granules with newly phagocytosed particles, with the purpose of carrying out oxygen-dependent intracellular killing of phagocytosable microorganisms (24). The lack of expression of NADPH oxidase components on crystalloid granules lends further support to the suggestion that eosinophils do not generate significant intracellular O_2^- during respiratory burst, and instead directs O_2^- towards extracellular regions. Although translocation of Rac to the cell membrane was not evident in Western blot analysis, it was detectable in confocal

microscopy analysis, suggesting that the latter technique may be substantially more sensitive to translocation events than Western blot. Even when Rac2 translocation was shown in PMA-stimulated guinea pig eosinophils in our previous report (19), the quantity translocated was barely detectable by immunoblot analysis.

The distinct pattern of Rac immunoreactivity in activated eosinophils *in vitro* was observed in *ex vivo* sputum samples from asthmatic patients. Rac immunofluorescence in normal sputum eosinophils was less than that of asthmatic cells, and did not exhibit a peripheral membrane pattern. In contrast, sputum eosinophils from asthmatic patients exhibited intense Rac immunofluorescence around the cell membrane, suggesting that these cells were stimulated and were actively releasing O_2^- into the tissues and airways. These observations indicate that sputum eosinophils in unstable asthma may be activated by *in vivo* stimuli to produce extracellular O_2^- and contribute to oxidant-mediated tissue injury. These novel observations indicate that eosinophil respiratory burst may be important in the pathogenesis of asthma.

In conclusion, eosinophils appear to assemble NADPH oxidase similarly to neutrophils at the level of molecular complex formation, while intracellular distribution of NADPH oxidase may differ significantly between these two cell types. This divergence, reflected in a predominantly plasma membrane association of Rac with p22*phox* in eosinophils versus a mainly intracellular location in neutrophils, may parallel distinct functional roles that these two cell types have in innate immunity. Thus, our findings suggest that sputum eosinophils from individuals with unstable asthma may be activated to produce more extracellular O_2^- than neutrophils, with the potential to induce tissue damage and contribute to the pathogenesis of this disease. Neutrophils may

produce comparatively less extracellular O_2^- in sputum from these individuals. The differential manner of NADPH oxidase assembly in these cells may have important implications for determining the activation status of airway eosinophils and, ultimately, treatment of oxidant-mediated tissue injury in asthma.

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Chapter 3

Anaplasma phagocytophilum *utilizes multiple host evasion mechanisms to thwart NADPH oxidase-mediated killing during neutrophil infection*

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D. Abdel Latif contribution to this study is as follows:

- Double labelling, confocal microscopy and image capturing of granulocytes
- Wrote the methods section for confocal microscopy section

Abstract

Anaplasma phagocytophilum, the etiologic agent of human anaplasmosis, is a bacterial pathogen that specifically colonizes neutrophils. Neutrophils utilize the NADPH oxidase complex to generate superoxide (O_2^-) and initiate oxidative killing of microorganisms. *A. phagocytophilum*'s unique tropism for neutrophils, however, indicates that it subverts and/ or avoids oxidative killing. We therefore examined the effects of A. *phagocytophilum* infection on neutrophil NADPH oxidase assembly and reactive oxygen species (ROS) production. Following neutrophil binding, *Anaplasma* invasion requires at least 240 minutes. During its prolonged association with the neutrophil plasma membrane, A. *phagocytophilum* stimulates NADPH oxidase assembly as indicated by increased cytochrome *b*$_{558}$ mobilization to the membrane, as well as co-localization of Rac and $p22^{phox}$. This initial stimulation taxes the host neutrophil's finite oxidase reserves as demonstrated by time- and bacterial dose-dependent decreases in secondary activation by N-formyl-methionyl-leucyl-phenylalanine (FMLP) or phorbol myristate acetate (PMA). This stimulation is modest, however, and does not diminish oxidase stores to nearly the extent as *Escherichia coli*, serum-opsonized zymosan (OpZ), FMLP, or PMA. Despite the apparent activation of NADPH oxidase, no change in ROS-dependent chemiluminescence is observed upon the addition of A. *phagocytophilum* to neutrophils, thereby indicating the bacterium may scavenge exogenous O_2^-. Indeed, A. *phagocytophilum* rapidly detoxifies O_2^- in a cell-free system. Once internalized, the bacterium resides within a protective vacuole that excludes $p22^{phox}$ $gp91^{phox}$. Thus, *A. phagocytophilum* employs at least two strategies to protect itself from neutrophil NADPH-oxidase mediated killing.

Introduction

Human anaplasmosis (formerly human granulocytic ehrlichiosis) is an emerging, vector-borne infection in the United States and Europe (8, 10, 15). The causative agent is *Anaplasma phagocytophilum*, an obligate intracellular bacterium that naturally cycles between ticks of the *Ixodes ricinus* complex and mammalian hosts (8, 15, 16). Humans are occasional inadvertent hosts. Following tick inoculation, human anaplasmosis initially presents with non-specific symptoms including fever, chills, malaise, and myalgia. More distinguishing manifestations include leukopenia, thrombocytopenia, and elevated levels of C-reactive protein and hepatic transaminases. The hallmark manifestation, however, is *A. phagocytophilum* colonization of polymorphonuclear leukocytes. Severe, potentially fatal complications can result and include prolonged fever, shock, pneumonitis, and increased susceptibility to opportunistic infections (8, 15). Neutrophils represent an initial line of defense against invading microorganisms. Individuals suffering from deficiencies in neutrophil numbers or function suffer from recurring, life-threatening infections and evidence the importance these phagocytes play in combating microbial pathogens (4). A primary means by which neutrophils destroy bacteria and fungi is through the production of toxic oxygen intermediates derived from superoxide O_2^- (34). Furthermore, the pH change within the phagosome resulting from the O_2^- influx triggers a potassium-dependent release of proteases that, in cooperation with hydrogen peroxide (H_2O_2) and hypochlorous acid, destroy ingested microorganisms (33, 34). The tightly controlled, multi-component NADPH oxidase complex is responsible for most of the inducible O_2^- production in activated neutrophils. The oxidase is unassembled in resting neutrophils and consists of membrane and cytosolic components. Cytochrome

b$_{558}$ is a membrane-spanning heterodimer composed of two subunits, gp91^{phox} and p22^{phox}, localized in the plasma membrane (approximately 5%) and in the membranes of mobilizable specific granules and secretory vesicles (approximately 95%) (12, 34). Cytochrome *b*$_{558}$ becomes expressed on the phagolysosome membrane and on the cell surface upon vesicular/ granular fusion with the larger membrane systems during enzyme activation. Concurrent with this event, the cytosolic components, Rac2, p40 ^{phox}, p47^{phox}, and p67^{phox} translocate and unite with cytochrome *b*$_{558}$ in the membrane to form the active enzyme complex, which transfers electrons from cytosolic NADPH to intraphagosomal or extracellular molecular oxygen, thereby generating O_2^- (34).

A. phagocytophilum's unique ability to invade and flourish within the hostile intra-neutrophil environment suggests that it employs mechanisms for avoiding and/ or subverting the normal microbicidal strategies of its host cell. Indeed, neutrophils from patients with human anaplasmosis, experimentally infected mice, or long term-infected HL-60 cells exhibit deficiencies in NADPH oxidase activation (2, 7, 37). This functional defect associated with established *A. phagocytophilum* infection is attributable to the fact that the bacterium inhibits transcription of genes encoding two key oxidase components, gp91^{phox} and Rac2 (2, 7). However, neutrophils emerge from the bone marrow fully armed with each of the oxidase components preformed and ready to assemble upon stimulation (34). As such, studies have demonstrated that neutrophils exhibit diminished production of ROS following *A. phagocytophilum* infection, although the time frame in which this occurs is a matter of dispute (11, 28). Furthermore, though possible mechanisms by which this phenomenon occurs have been postulated (29), evidence remains inconclusive. We therefore correlated the effects of *A. phagocytophilum* on

neutrophil ROS production, as well as the assembly and membrane localization of NADPH oxidase components, at various time points during infection. We also determined whether the bacterium is capable of directly scavenging O_2^- in a cell-free system. This study provides further insight into the means by which *A. phagocytophilum* combats O_2^--mediated killing by its target mammalian cell.

Materials and Methods

Cultivation of *A. phagocytophilum* strains and isolation of host-cell free bacteria

Unless otherwise noted, *A. phagocytophilum* strain NCH-1 (7) was used in these studies. *A. phagocytophilum* strain HZ was kindly provided by Drs. Ralph Horowitz of New York Medical College (Valhalla, NY) and Yasuko Rikihisa of Ohio State University (Columbus, OH). *A. phagocytophilum* strains MD, 96HE 158, and 99HE 4 were generously provided by Dr. J. Stephen Dumler of Johns Hopkins University. Each strain was cultivated in HL-60 cells and host-cell free *A. phagocytophilum* were prepared as previously described (7).

Isolation of neutrophils

Human neutrophils were isolated from peripheral blood from healthy donors by centrifugation through an equal volume of Polymorphprep (Axis-Shield; Greiner Bio-One, Frickenhausen, Germany) at 470 *g* for 30 min. The resulting neutrophil band was removed by aspiration and mixed with equal volumes of 0.45% NaCl and RPMI 1640 (Invitrogen, Carlsbad, CA)/ 0.5 mM EDTA to re-establish isotonic conditions. The cells were centrifugated at 210 *g* for 10 min, washed twice with RPMI 1640/ 0.5 mM EDTA, resuspended in RPMI 1640, and enumerated using a hemacytometer.

Assessment of *A. phagocytophilum* binding and invasion

Five hundred µl suspensions of 1 x 10^6 neutrophils per ml of RPMI 1640 were added to individual wells of a 24-well Ultra Low Attachment plate (Corning Inc.; Corning, NY). To these were added suspensions of host cell-free *A. phagocytophilum* that had been liberated from 2.5 x 10^6 highly (> 90%) infected HL-60 cells. At 5, 15, 30, 60, 120, and 240 minutes post-infection (mpi), aliquots were removed and washed twice with

RPMI 1640/ 0.5 mM EDTA. Two hundred µl of 5 x 10^5 neutrophils/ ml in RPMI 1640/ 20% FCS were centrifugated at 70 *g* for 2 min using a Cytospin 4 centrifuge (Thermo Electron, Pittsburgh, PA). Cytsopins were fixed in 2% paraformaldehyde (PFA)/ PBS and screened using an indirect immunofluroescence assay to determine the percentage of infected neutrophils and the number of bacteria per infected cell as previously described (7).

To assess the rate at which bound *A. phagocytophilum* are internalized, viable host cell-free bacteria were fluoresceinated with Celltracker Green (Molecular Probes, Eugene, OR) as previously described (6, 39). Celltracker Green-labeled bacteria were added to neutrophils at ratios of 1, 5, and 50 per cell. Cells were incubated at 37 °C in 5% CO_2 for 1, 2, or 4 h. At the appropriate time post-infection, cells were washed twice with RPMI 1640/ 0.5 mM EDTA, resuspended in 500 µl 1X PBS/ 1% FCS/ 1% PFA and analyzed by flow cytometry using a FACScalibur (Becton Dickinson; San Diego, CA). Data were analyzed using Cellquest (Becton Dickinson). Bacterial internalization was inferred as a decrease in CellTracker Green fluorescence over time.

Assessment of neutrophil O_2^- production

Suspensions of 100,000 neutrophils in 100 µl of RPMI 1640 were added to luminometer cuvettes (Promega, Madison, WI). To these was added 100 µl of RPMI 1640 alone or RPMI 1640 containing host cell-free *A. phagocytophilum* followed by incubations of 0, 60, 120, or 240 min at 37 °C in 5% CO_2. In some cases, *A. phagocytophilum* were incubated with either 20% autologous serum or rabbit polyclonal antiserum against *A. phagocytophilum* prior to being added to neutrophils. In some cases, zymosan A particles from *Saccaromyces* cerevisiae (Sigma) that had been opsonized with

autologous serum (OpZ) or *E. coli* were added to neutrophils at ratios of 10 particles or bacteria per cell, respectively. At the appropriate time post-infection or post-addition of stimuli, 100 µl of Diogenes (National Diagnostics, Atlanta, GA) was added. Diogenes is a chemiluminescent substrate that is highly specific for O_2^- and is unaffected by the presence of H_2O_2. In some cases, this was followed by the addition of either PMA (100 ng/ml; Sigma) or FMLP (1 µM; Sigma). For uninduced samples, vehicle alone was added. Changes in Diogenes chemiluminescence (DCL) were recorded in total relative light units (RLU) using a TD-20/20 luminometer (Turner Designs; Promega).

Assessment of *A. phagocytophilum* infection, surface-associated gp91*phox*, and host cell size by flow cytometry

Celltracker Green-labeled bacteria were added to neutrophils at ratios of 0.3, 3, and 30 per cell in a final volume of 1 ml RPMI 1640 in individual wells of a 24-well Ultra Low Attachment plate and incubated at 37 °C in 5% CO_2 for 1, 2, or 4 h. At the appropriate time post-infection, cells were washed twice with RPMI 1640/ 0.5 mM EDTA and incubated with 5 µg/ ml anti-CD16/ CD32 and 1 µg/ ml mouse IgG_1 isotype control (both from BD Pharmingen; San Diego, CA) in 100 µl 1X PBS/ 1% fetal bovine serum (FBS) for 30 min on ice. The cells were successively screened with 3 µg/ ml mAb 7D5 (a kind gift from Dr. Dirk Roos of the University of Amsterdam (34)) and Cy5-conjugated anti-mouse IgG_1 (BD Pharmingen), each in 1X PBS/ 1% FBS for 30 min on ice. After each antibody incubation, cells were washed with 1 ml 1X PBS/ 1% FCS. Cells were resuspended in 500 µl 1X PBS/ 1% FCS/ 1% PFA and analyzed by flow cytometry.

Double labeling and confocal laser scanning microscopy.

Neutrophils were isolated and incubated in the presence or absence of *A. phagocytophilum* as described above. To ensure that conditions were optimal for infection, an equivalent aliquot of bacteria was labeled with CellTracker Green and used to infect a parallel sample of neutrophils, which was subsequently examined by flow cytometry. HL-60 lysate was added as a control. PMA or vehicle alone was added. Cytsopins were prepared and fixed as described above and double labeled for Rac and $p22^{phox}$ using anti-Rac mAb (Transduction Laboratories; BD Biosciences; San Diego, CA) and anti-$p22^{phox}$ mAb 44.1 (kindly provided by Dr. Mark Quinn, Montana State University, [Bozeman, MT]), respectively. Labeled samples were examined and images were collected as previously described (23, 24).

Assessment of cell-free O_2^- and urate production.

Xanthine (100 µM; Sigma) in 50 mM KH_2PO_4 was incubated in the presence or absence of 0.125 mg/ ml superoxide dismutase (SOD; Sigma). Titrated concentrations of host cell-free *A. phagocytophilum* in Hank's Balanced Salt Solution were added. In some cases, *A. phagocytophilum* were either heated to 60 °C or treated with Pronase E (Sigma; Saint Louis, MO) at 37 °C for 20 min prior to being added. HL-60 lysate components were added to control samples. Diogenes was added followed by the addition of xanthine oxidase (25 µM; Sigma). O_2^- was indirectly measured as the change in DCL every 10 sec for a period of 3 min. For measuring uric acid production, the assay conditions were repeated except that the change in A_{290} was recorded.

Electron microscopy

Neutrophils were incubated with host cell-free *A. phagocytophilum* for 240 min as described above, followed by washing with PBS to removed unattached bacteria. Cells were initially fixed for 1 h at room temperature with 4% PFA in 0.25 M Hepes, pH 7.4 followed by fixation overnight at 4 °C with 8% PFA in 0.25 M Hepes, pH 7.4. Samples were imbedded in gelatin, sectioned, and screened as previously described (18). The final concentrations of mAbs 7D5 and 44.1 were 30 μg/ ml and 3.25 μg/ml, respectively.

Statistical analyses

Statistical analysis was performed using the Prism 4.0 software package (Graphpad; San Diego, CA). If Analysis of Variance (ANOVA) indicated a group difference ($p < 0.05$) then Tukey's test was used to test for a significant difference among groups.

Results

A. phagocytophilum exhibits prolonged association with the neutrophil outer membrane during invasion.

To properly assess the effects of *A. phagocytophilum* infection on neutrophil ROS production, it was first essential to determine the kinetics by which the bacterium binds and invades its host cell. Neutrophils were incubated with host cell-free *A. phagocytophilum* that had been liberated from a five-fold excess of highly infected HL-60 cells. At 5, 15, 30, 60, 120, and 240 mpi, the neutrophils were examined by immunofluorescence microscopy for the percentage of infected cells and the number of *A. phagocytophilum* per infected cell. Under the conditions employed, 30 to 60 min of incubation was required for at least 70.5 + 4.6% of neutrophils to be bound with a minimum of 2.9 + 0.6 bacteria per cell (Figure 3.1, Panels A and B). At earlier time points, less than 38.3 + 2.8% of cells were infected with less than 2 bacteria per cell. Achieving greater than 90% infection, which would be necessary to reliably assess the effect of *A. phagocytophilum* infection on NADPH oxidase function, required an incubation period of 240 min. The relatively slow binding kinetics are likely dependent on the cells being in suspension and are not likely reflective of the bacterium's avidity for its host cell, as it is well established that *A. phagocytophilum* tenaciously binds neutrophils (6, 19, 20, 39).

To assess the rate at which bound *A. phagocytophilum* enter neutrophils, host cell-free *A. phagocytophilum* were fluoresceinated using the viable dye Celltracker Green, which has proven effective for tracing *A. phagocytophilum* binding and infection (6, 20, 39), and added to neutrophils at ratios of 1, 5, and 50 bacteria per cell for periods of 60,

120, or 240 min. When assessed by flow cytometry, Celltracker Green fluorescence intensity correlated directly with bacterial load (Figure 3.1C). Since the fluorescence intensity of CellTracker Green-labeled *A. phagocytophilum* is stable for at least 48 h (J. A. Carlyon and E. Fikrig, unpublished observation), the degree of *Anaplasma* internalization was indirectly assessed as a decrease in fluorescence over the duration of the time course. Maximal internalization was not achieved until 240 mpi. This indicates that internalization of bound *A. phagocytophilum* is a lengthy process. Based on our observations, neutrophils were infected for periods of 60, 120, or 240 min for subsequent NADPH oxidase assays.

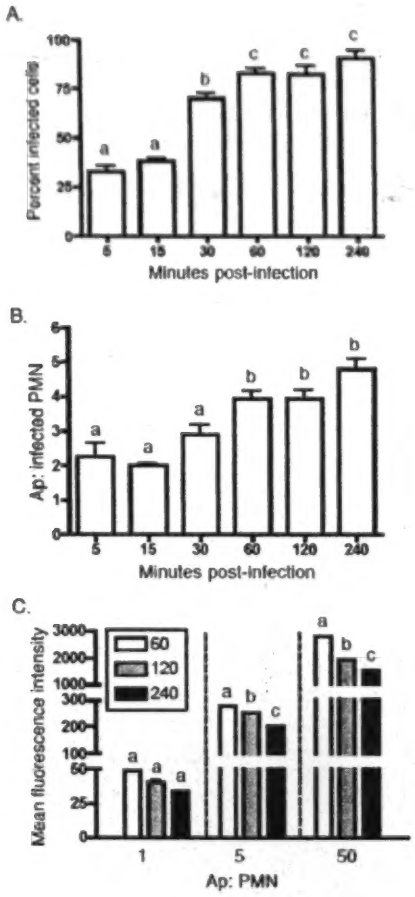

Figure 3.1. *A. phagocytophilum* binding and invasion of neutrophils.
Host cell-free *A. phagocytophilum* were added to neutrophils as described in Materials and Methods. At the indicated times post-infection, aliquots of neutrophils were examined by immunofluorescence microscopy for the percentage of infected cells (A) and the number of *A. phagocytophilum* per infected neutrophil (B). Results are presented as the mean + SD of five independent counts of at least 100 cells per time point. (C) To indirectly assess the rate at which bound *A. phagocytophilum* enter neutrophils, host-free *A. phagocytophilum* were fluoresceinated with CellTracker Green, added to neutrophils at approximate ratios of 1, 5, and 50 bacteria per neutrophil (Ap: PMN) for 60 (_), 120 (■), or 240 (■) min, and examined by flow cytometry. Results are presented as the mean (+ SD) of assays performed in triplicate. Statistical significance ($p < 0.05$) was determined using ANOVA followed by Tukey's test. Mean values denoted by different letters are significantly different. Results are representative for 3 independent studies. (Figure 3.1 was generated by J. A. Carlyon)

***A. phagocytophilum* infected neutrophils exhibit reduced ROS production.**

To study the effects *A. phagocytophilum* infection has on NADPH oxidase activity, neutrophils were incubated with titrated concentrations of host cell-free *A. phagocytophilum* strain NCH-1 or HL-60 lysate as a negative control for periods of 60, 120, or 240 min. At the appropriate time post-incubation, Diogenes substrate was added followed by the addition of FMLP or PMA. ROS production was monitored kinetically as the change in DCL. In the absence of exogenous stimuli, *A. phagocytophilum* elicited little to no increase in DCL regardless of concentration. In contrast to controls, cells incubated with NCH-1 demonstrated decreases in PMA activation that correlated directly with bacterial load and duration of infection (Figure 3.2A). The greatest inhibition, which was marked by a 35.1% reduction in ROS production, was observed at 240 mpi and occurred with a ratio of 30 *A. phagocytophilum* per neutrophil.

Interestingly, neutrophils that had been incubated with 30 bacteria per cell displayed a 57.5% increase in ROS when stimulated with FMLP 60 mpi, while those incubated for 120 and 240 min demonstrated decreases of 66.6% and 25.2% upon stimulation, respectively (Figure 3.2B). A similar, but delayed response to FMLP was observed for neutrophils incubated with 10 *Anaplasma* per cell. While there was little difference in ROS production between infected and uninfected control cells at 60 mpi, a 33.1% increase was observed at 120 mpi, which nearly subsided by 240 mpi. Ratios of 1 or less bacteria per neutrophil exhibited no difference in either PMA- or FMLP-mediated ROS from uninfected controls (data not shown). Also, results observed at 30 mpi exhibited little difference from those observed at 60 mpi (data not shown). Collectively, these data demonstrate that *A. phagocytophilum* infection reduces NADPH oxidase

responsiveness in a time- and dose-dependent manner and suggest that *Anaplasma* may even stimulate oxidase activity during the early phase of infection.

The initial induction and subsequent abrogation of the neutrophil respiratory burst by *A. phagocytophilum* infection that we observed are consistent with a report by Choi and Dumler (11), but differ from the rapid, absolute inhibition suggested by Mott *et al* (28). Because of this inconsistency, we extended our analyses to include *A. phagocytophilum* strain HZ, which was used in their study (28). In addition, we also performed our analyses using strains MD, 96HE 158, and 99HE 4. Results obtained with all four strains reproduced those observed for NCH-1 (data not shown). Due to the consistency in our observations obtained using five different *A. phagocytophilum* strains, we chose strain NCH-1 for the remaining studies.

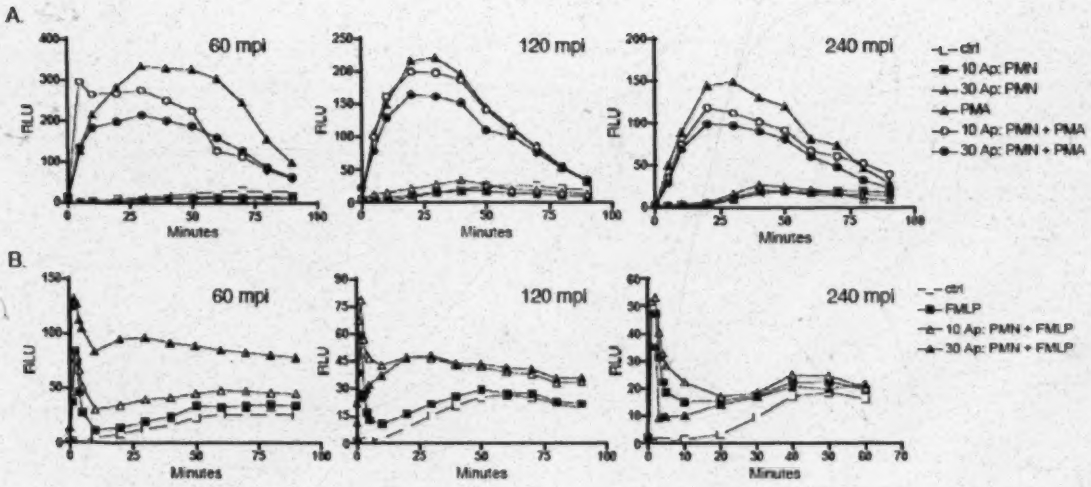

Figure 3.2. *A. phagocytophilum* infected neutrophils exhibit reduced ROS production.

Host-cell free *A. phagocytophilum* were added to 100,000 neutrophils at approximate ratios of 10 and 30 Ap: PMN and incubated for 60, 120, or 240 min. Neutrophils incubated with HL-60 lysate served as a control. At various mpi, PMA (A) or FMLP (B) was added and ROS production was indirectly measured as the change in DCL over time. Data are presented in relative luminometer units (RLU). Results are representative for at least 10 independent experiments. (Figure 3.2 was generated by J. A. Carlyon)

Exposure to *A. phagocytophilum* yields little to no change in detectable ROS levels.

Neutrophils exhibit reduced NADPH oxidase responsiveness post-*A. phagocytophilum* infection. However, our data also suggests that initial interaction with the bacterium might elicit such activity. Moreover, though several studies have examined O_2^- production by *A. phagocytophilum*-infected myelocytic cells (2, 7, 11, 28, 37), none have assessed the immediate effect the bacterium has on NADPH oxidase activity. We therefore examined neutrophil ROS production immediately following the addition of *A. phagocytophilum* at ratios of 1, 10, or 30 bacteria per cell. Relative to uninfected controls, very little change in DCL was observed for neutrophils incubated with *A. phagocytophilum*, regardless of bacterial concentration (Figure 3.3A). In sharp contrast, however, neutrophils yielded significantly higher ROS levels upon exposure to OpZ, which would have presumably activated NADPH oxidase by Fc receptor (FcR)-mediated phagocytosis (1).

Current evidence suggests that *A. phagocytophilum*, unlike many other bacteria species, are not internalized into neutrophils or HL-60 cells by phagocytosis. Instead, they actively enter their mammalian host cells via a process involving caveolae and endocytosis (3, 25, 27, 38, 40). Consistent with this, *Anaplasma* opsonized by rabbit polyclonal *A. phagocytophilum* antiserum, which presumably would have been internalized by FcR-mediated phagocytosis, elicited an oxidase response several fold higher than that of *Anaplasma* pre-incubated with either normal human serum or no serum (Figure 3.3B).

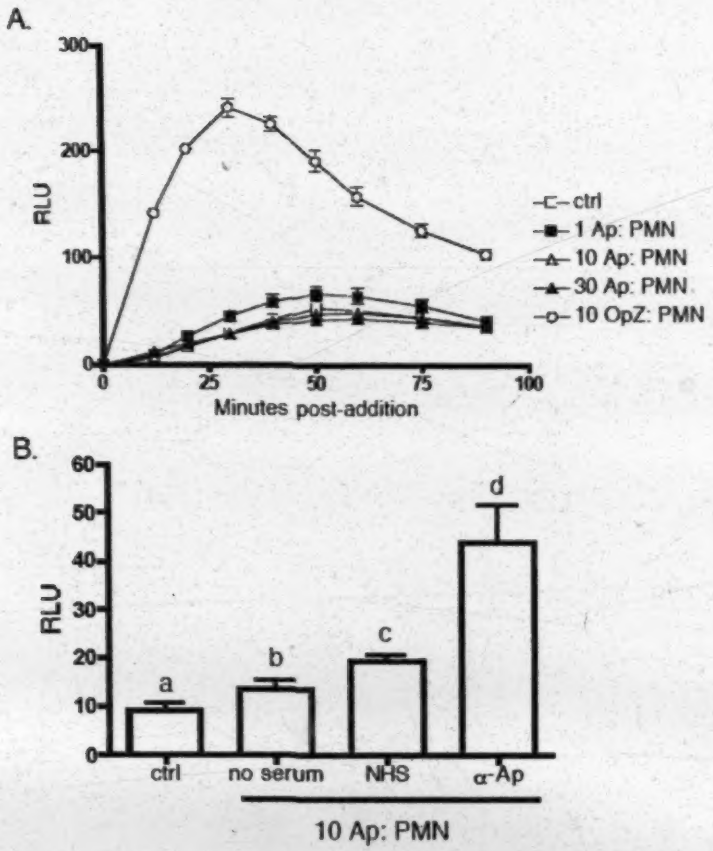

Figure 3.3. Induction of neutrophil reactive oxygen species production by *A. phagocytophilum*.
(A) Host cell-free *A. phagocytophilum* (Ap; ratios of approximately 1, 10, or 30 per cell) or OpZ (10 per cell) were added to samples of 100,000 neutrophils and ROS production was indirectly assessed as the change in DCL at 10 min intervals over a 90 min time course. (B) Non-opsonized host cell-free *A. phagocytophilum* or bacteria that had been incubated with either normal human serum (NHS) or rabbit polyclonal anti- *A. phagocytophilum* antiserum were added to neutrophils at 10 Ap: PMN. ROS was recorded as described for (A). Neutrophils alone served as a control for both experiments. Data are presented as the mean (+ SD) maximum change in RLU per time point. Assays were performed in triplicate. Statistical significance ($p < 0.05$) was determined using ANOVA followed by Tukey's test. Mean values denoted by different letters are significantly different. Results are representative for 3 independent experiments. (Figure 3.3 was generated by J. A. Carlyon)

Initial exposure to *A. phagocytophilum* mobilizes neutrophil NADPH oxidase reserves.

Secondary activation of neutrophils results in reduced O_2^- generation as their NADPH oxidase stores are finite and cannot be replenished (1, 12, 34). *A. phagocytophilum*-infected neutrophils demonstrate lowered NADPH oxidase responsiveness to both FMLP and PMA stimulation that is maximal 240 min post-infection. We therefore assessed whether inhibition of ROS generation is specific for *A. phagocytophilum* infection or merely the consequence of the bacterium stimulating oxidase mobilization during binding and invasion. Neutrophils were initially stimulated with host cell-free *A. phagocytophilum*, *E. coli*, OpZ, FMLP, or PMA. Four h post-stimulation, the neutrophils were re-stimulated with either FMLP or PMA and the resulting change in DCL was observed. Uninfected cells receiving no stimuli at either time point served as negative controls, while those exposed to stimuli at 4 h only served as positive controls. *E. coli*, OpZ, and PMA each elicited respiratory bursts substantial enough to exhaust neutrophil oxidase reserves, as little or no ROS were released upon secondary stimulation by FMLP or PMA (Figure 3.4). Consistent with our previous observations, *A. phagocytophilum*-infected neutrophils displayed a dose-dependent reduction in ROS release upon secondary activation by FMLP (Figure 3.4A). However, this reduction was not very pronounced. Only at an excessively high concentration of 100 *Anaplasma* per cell did the drop in secondary FMLP stimulation begin to approach that yielded by pre-incubation with *E. coli*, OpZ, FMLP, or PMA.

Re-stimulation by PMA was modestly affected by *A. phagocytophilum* infection for all bacterial concentrations (Figure 3.4B). The discrepancy in secondary activation profiles

between FMLP and PMA is likely due to the difference in the duration over which they activate NADPH oxidase. While FMLP activation is rapid and transient, PMA irreversibly activates proteins containing a C1 domain, principally protein kinase C (1). Subjecting *Anaplasma* to either heat or pronase treatment eliminated bacterial binding to neutrophils, which consequently did not alter the NADPH oxidase response upon secondary PMA activation (data not shown). Consistent with published data, initial stimulation with FMLP primed neutrophils for a strong, secondary response to PMA (21). The secondary activation profiles of *A. phagocytophilum* infected neutrophils strongly suggest physical interaction with the bacterium stimulates NADPH oxidase activation, though to a considerably lesser degree than *E. coli*, phagocytic particles, or soluble stimuli. However, *A. phagocytophilum* elicits very little to no change in DCL, which presents an interesting paradox warranting further investigation.

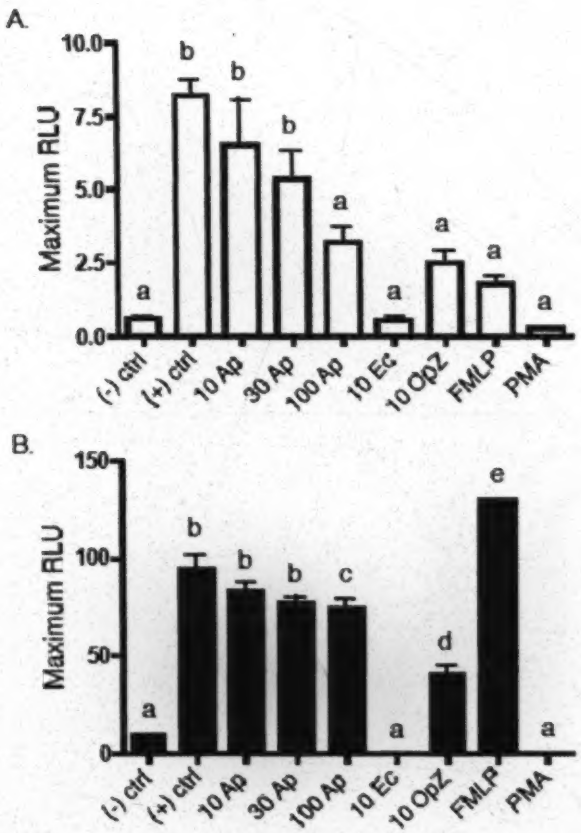

Figure 3.4. Neutrophil re-stimulation assay.
Samples of 100,000 neutrophils were initially exposed to host cell-free *A. phagocytophilum* (Ap; 10, 30, or 100 per cell), *E. coli* (Ec; 10 per cell), OpZ (10 per cell), FMLP, or PMA. Four h later, the neutrophils were re-stimulated with either FMLP (A) or PMA (B) and ROS was indirectly measured as the change in DCL over 60 min. Neutrophils receiving no stimulation at either 0 or 4 h served as negative controls, while those receiving stimulation only at 4 h served as positive controls. Data are presented as the mean (+ SD) maximum change in RLU. Assays were performed in triplicate. Statistical significance ($p < 0.05$) was determined using ANOVA followed by Tukey's test. Mean values denoted by different letters are significantly different. Results are representative for 3 independent experiments. (Figure 3.4 was generated by J. A. Carlyon)

***A. phagocytophilum* stimulates cytochrome *b*$_{558}$ aggregation at the neutrophil plasma membrane and cell size increase.**

Our re-stimulation data suggests that *A. phagocytophilum* interaction results in neutrophil activation and NADPH oxidase mobilization. This is contrasted, however, by our observation that addition of the bacterium to neutrophils results in little to no change in DCL. Therefore, we shifted our focus to directly gauge NADPH oxidase assembly in the context of *A. phagocytophilum* infection. We first assessed localization of cytochrome *b*$_{558}$ to the neutrophil plasma membrane in response to *A. phagocytophilum*. Celltracker Green-labeled *A. phagocytophilum* were added to neutrophils at ratios of 0.3, 3, and 30 bacteria per cell for periods of 60, 120, or 240 min. The infected neutrophils were gated according to CellTracker Green fluorescence and examined for levels of plasma membrane-associated cytochrome *b*$_{558}$ based on their ability to be recognized by mAb 7D5, which targets a surface-exposed epitope of gp91^{phox}. This epitope is only accessible when gp91^{phox} is complexed with p22^{phox}, thereby forming the cytochrome *b*$_{558}$ heterodimer (5, 30). *A. phagocytophilum* stimulated cytochrome *b*$_{558}$ association with the neutrophil plasma membrane in a time- and dose-dependent manner (Figure 3.5A).

Another phenomenon associated with neutrophil activation is an increase in cell size (14, 17, 22, 31), which can be directly measured by flow cytometry as an increase in forward scatter. The forward scatter of infected cells increased directly with bacterial load and duration of infection (Figure 3.5B), thereby providing further evidence that *A. phagocytophilum* interaction leads to host cell activation. Neutrophils incubated with HL-60 lysate demonstrated no increase in either cytochrome *b*$_{558}$ or forward scatter (data not shown). These data confirm that *A. phagocytophilum* activates its host cell during

infection and stimulates the localization of cytochrome *b*$_{558}$ to the neutrophil plasma membrane, which is an integral step in NADPH oxidase assembly.

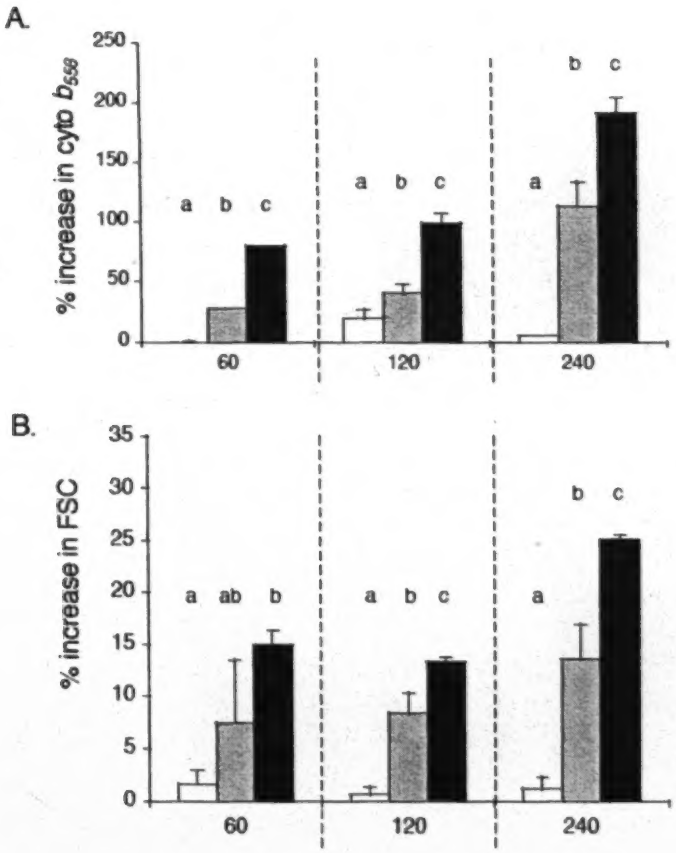

Figure 3.5. *A. phagocytophilum* interaction results in increased cytochrome b_{558} association with the neutrophil plasma membrane and increased host-cell size. Viable host-cell free *A. phagocytophilum* were fluorescently labeled using CellTracker Green and added to neutrophils at approximate Ap: PMN ratios of 0.3 (◯), 3 (■), and 30 (■) for periods of 60, 120, and 240 min. (A) At the appropriate time post-addition of bacteria, infected neutrophils were assessed by flow cytometry for plasma membrane levels of cytochrome b_{558} as determined by mAb 7D5 recognition. (B) Infected cells were also examined for changes in their forward scatter properties as an indication of cell size variation. Statistical significance ($p < 0.05$) was determined for each set of means per post-infection time point using ANOVA followed by Tukey's test. Mean values denoted by different letters are significantly different. Results are representative for 3 independent experiments. (Figure 3.5 was generated by J. A. Carlyon)

***A. phagocytophilum* stimulates co-localization of $p22_{phox}$ and Rac2.**

Our flow cytometric data suggest that interaction of *A. phagocytophilum* with its host neutrophil elicits plasma membrane association of cytochrome *b558*, with the highest degree of association occurring at 240 mpi. As a means of determining the bacterium's effect on the assembly of additional oxidase components in the presence and absence of secondary stimulation, *A. phagocytophilum*-infected neutrophils were directly visualized for intracellular levels and co-localization of $p22^{phox}$ and Rac using confocal microscopy following the addition of PMA or vehicle alone. Neutrophils were incubated with *A. phagocytophilum* at a ratio of 3.3 + 0.6 bacteria per cell (as determined by immunofluorescent microscopy) for 240 min. Higher ratios resulted in considerable, irreversible neutrophil clumping which precluded our ability to examine individual cells. Neutrophils incubated with HL-60 lysate components served as a negative control.

Control neutrophils exhibited very diffuse staining for both $p22^{phox}$ and Rac and demonstrated no co-localization (Figure 3.6, Panels A-D). Upon PMA-stimulation, the staining patterns for each changed dramatically, becoming considerably more intense and patchy (Figure 3.6, Panels I-L). Such patterns are consistent with NADPH oxidase assembly (23, 24). Moreover, significant co-localization occurred as indicated by a high distribution of yellow to yellow-orange areas throughout the cells, providing further evidence of oxidase formation. *A. phagocytophilum*-infected cells exhibited considerable degrees of patchy $p22^{phox}$ and Rac staining, although the patterns were not as intense as those associated with PMA stimulation (Figure 3.6, Panels E-H). Also, at least some degree of Rac and $p22^{phox}$ co-localization occurred in infected cells, indicating the bacterium evokes at least minor levels of oxidase assembly. This data is consistent with

our flow cytometric studies. Upon PMA stimulation, however, infected neutrophils demonstrated complete redistribution of both Rac and $p22^{phox}$ to the plasma membrane with minute patches of co-localization (Figure 3.6, Panels M-P). This is not surprising as PMA promotes plasma membrane enrichment of NADPH oxidase components (13). Therefore, the combination of stimuli would be expected to induce greater oxidase mobilization than either alone. The results presented for individual cells in Figure 3.6 are representative for the majority of cells per each examined population (Figure 3.6, Panels Q-T). Furthermore, Z-stack analyses revealed that the co-localization and plasma membrane redistribution of Rac and $p22^{phox}$ observed for infected and PMA-activated, infected cells, respectively, were not restricted to a single focal plane (Figure 3.7). Collectively, our flow cytometric and confocal studies illustrate that *A. phagocytophilum* stimulates aggregation and assembly of at least 2 oxidase components, Rac and cytochrome b_{558}.

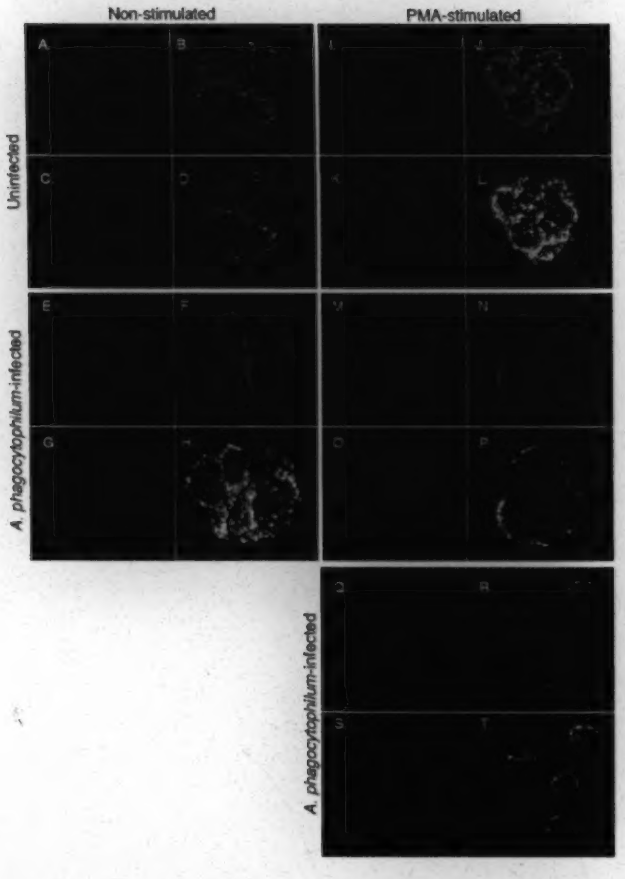

Figure 3.6. Confocal analysis of Rac and p22^{phox} co-localization in uninfected and *A. phagocytophilum*-infected neutrophils.
Neutrophils were incubated in the presence of host-cell free *A. phagocytophilum* for 240 min. Control neutrophils were incubated with HL-60 lysate components. At 240 mpi, infected neutrophils had 3.3 + 0.6 bacteria per cell. Infected and control neutrophils were stimulated with PMA for 5 min. Cytospins of each sample were double labeled for Rac and p22^{phox}, followed by DAPI nuclear counterstain (blue; C, G, K, O, and S). Rac immunofluorescence (A, E, I, M, and Q), shown in red, was overlaid with green p22^{phox} immunofluorescence (B, F, J, N, and R) to determine co-localization as indicated by yellow-orange color (D, H, L, P, and T). A-D, uninfected, non-stimulated neutrophils; E-H, *A. phagocytophilum*-infected, non-stimulated neutrophils, I-L, uninfected, PMA-stimulated neutrophils, M-P, *A. phagocytophilum*-infected, PMA-stimulated neutrophils; Q-T, lower magnification of infected, PMA-stimulated cells showing a population of cells in one field. Original magnification, X40. Results are indicative of 2 separate experiments. (Figure 3.6 was generated by D. Abdel Latif).

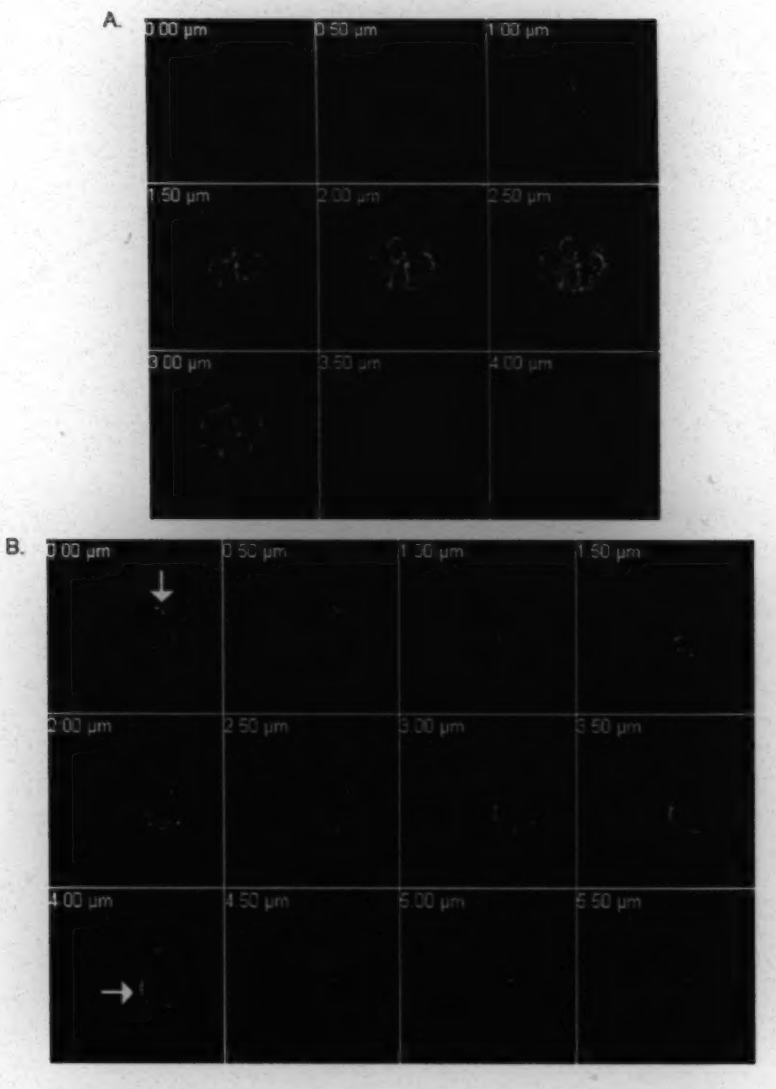

Figure 3.7. Z-sections of *A. phagocytophilum*-infected cells.
Cells from Figure 3.6 E-H and M-P are shown in 0.5 mm Z-sections to indicate membrane localization of Rac (red) and p22phox (green), with blue indicating nuclei. (A) Neutrophils infected with A. phagocytophilum, (B) A. phagocytophilum-infected, PMA-stimulated neutrophils. Cells were treated as described for Figure 3.6. Sections are shown from top to bottom of cells on cytospins. Arrows indicate co-localization of Rac and $p22^{phox}$ on the membrane of infected, PMA-stimulated cells. Original magnification, 40X. (Figure 3.7 was generated by D. Abdel Latif)

***A. phagocytophilum* reduces O_2^- produced in a cell-free system.**

Upon interaction with *A. phagocytophilum*, neutrophils exhibit very little to no change in DCL despite the assembly of at least 2 oxidase components. This raises the likelihood that *A. phagocytophilum* is able to directly detoxify O_2^-. We therefore investigated this possibility using a cell-free system in which xanthine oxidase is added to xanthine to rapidly generate uric acid with O_2^- and H_2O_2 as by-products. As previously stated, the DCL assay employed is highly specific for O_2^- and is unaffected by the presence of H_2O_2. Prior to the addition of xanthine oxidase, host cell-free *A. phagocytophilum* or HL-60 lysate components were added to xanthine at concentrations ranging from 3×10^4 to 3×10^6 bacteria, which would have corresponded to bacteria to neutrophil ratios ranging from 0.3 to 30, respectively. As a positive control for O_2^- detoxification, samples were also incubated in the presence of SOD. The addition of xanthine oxidase to xanthine rapidly produced O_2^- as inferred by a sharp rise in DCL that peaked 10 seconds post-addition (Figure 3.8A). This system, however, yielded no change in DCL in the presence of SOD. *A. phagocytophilum* inhibited cell-free O_2^- production in a dose-dependent manner. Concentrations of 3×10^5 and 3×10^6 bacteria markedly reduced, but did not abolish O_2^- generation, while a concentration of 3×10^4 exhibited little to no effect. This inhibition was specific for O_2^- as neither SOD nor *A. phagocytophilum* affected urate production (Figure 3.8B). The *A. phagocytophilum* mechanism responsible for rapidly scavenging exogenous O_2^- involves a heat-labile surface protein(s) as both Pronase E digestion and heat treatment eliminated the bacterium's ability to detoxify O_2^- (Figure 3.8C). Thus, while *A. phagocytophilum* apparently stimulates production of O_2^- by its target neutrophil, it is able to rapidly

scavenge the anion, which likely serves as a means for protecting itself during host cell binding and invasion.

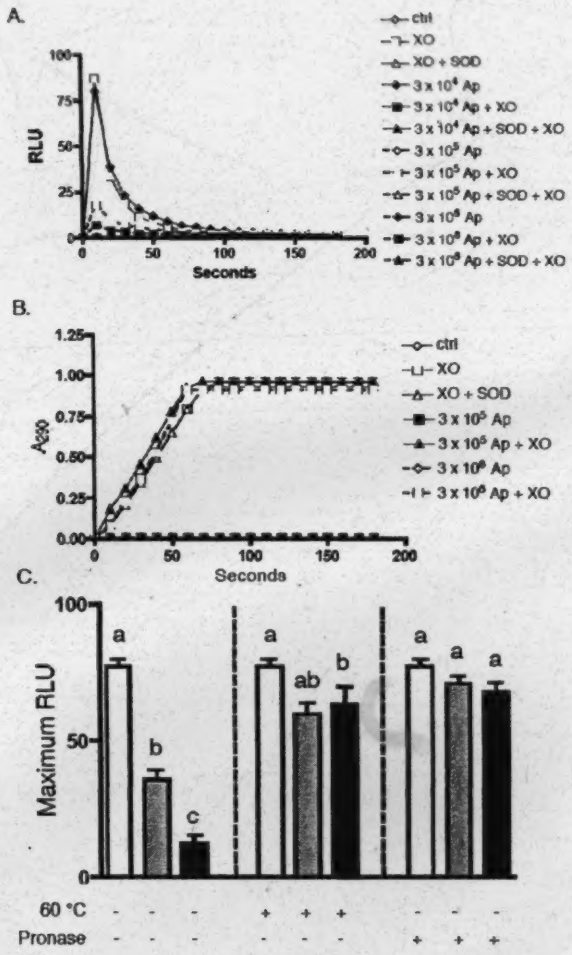

Figure 3.8. *A. phagocytophilum* detoxifies O_2^- produced in a cell-free system. Host cell-free *A. phagocytophilum* were added to xanthine at concentrations of 3 x 10^4, 3 x 10^5, and 3 x 10^6. HL-60 lysate components were added as controls. SOD was added to duplicate samples. (A) Upon the addition of xanthine oxidase, cell-free O_2^- production was measured as the resulting change in DCL every 10 sec over a 3 min period. Results are representative for 10 experiments with similar results. (B) Uric acid production was measured as the change in A_{290}. Results are representative for 4 separate experiments. (C) The assay was repeated using 0 (□), 3 x 10^5 (■), or 3 x 10^6 (■) untreated host-cell free *A. phagocytophilum* or that which had either been heated to 60 °C or incubated with Pronase E at 37 °C for 20 min. Statistical significance ($p < 0.05$) was determined for each set of means per treatment using ANOVA followed by Tukey's test. Mean values denoted by different letters are significantly different. Results are representative for 5 independent experiments. (Figure 3.8 was generated by J. A. Carlyon)

***A. phagocytophilum* vacuole excludes gp91^{phox} and p22^{phox}.**

A. phagocytophilum binding and invasion stimulates cytochrome b_{558} recruitment to the neutrophil plasma membrane. The bacterium protects itself by scavenging released O_2^-. Two hundred and forty min post-infection, when the majority of *Anaplasma* would have been internalized, neutrophils retain the capability to mobilize NADPH oxidase to the plasma membrane and produce ROS. The intracellular fate of *A. phagocytophilum* with regards to NADPH oxidase mobilization is unknown. To assess whether NADPH oxidase components localize to the *A. phagocytophilum* vacuole, frozen sections of infected neutrophils were examined 240 mpi by immunoelectron microscopy using antibodies targeting gp91^{phox} and p22^{phox}. Consistent with previous observations, *A. phagocytophilum* infection results in the host cell's cytoplasm appearing more electron lucent (38). As shown in Figure 3.9, gold particles associated predominantly with secretory vesicles but not with the *A. phagocytophilum* parasitophorous vacuole. This is especially evident in Figure 3.9B, in which two p22^{phox}-labeled vesicles reside in close proximity to, but fail to fuse with the *Anaplasma* vacuolar membrane. Thus, once internalized, *A. phagocytophilum* resides within a protective compartment that excludes NADPH oxidase, thereby providing it with a safe haven from intracellularly derived ROS.

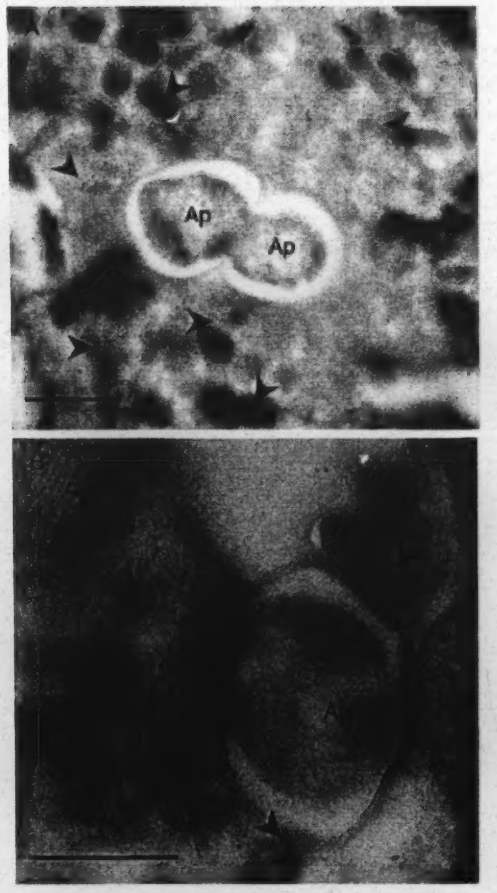

Figure 3.9. Neither gp91^{phox} nor p22^{phox} co-localize to the *A. phagocytophilum* vacuolar membrane.
Neutrophils were examined by immunoelectron microscopy 240 min post-*A. phagocytophilum* infection as described in Materials and Methods using mAbs directed against gp91^{phox} (A) and p22^{phox} (B). Arrowheads highlight representative gold particles. Ap, *A. phagocytophilum*; bar, 500 nm. Results are representative for several micrographs. (Figure 3.9 was generated by M. Pypaert)

Discussion

NADPH oxidase-mediated O_2^- production is a major means by which neutrophils kill bacteria. *A. phagocytophilum*, however, specifically invades and flourishes within these soldier phagocytes, indicating that it uses strategies for avoiding and/ or combating the respiratory burst. Indeed, this bacterium scavenges extracellular O_2^-. This likely serves to protect *A. phagocytophilum* during the initial steps of neutrophil invasion, which proceeds at a rather inefficient rate. During its prolonged association with the neutrophil plasma membrane, *A. phagocytophilum* stimulates cellular activation, assembly and membrane localization of at least 2 oxidase components, and subsequent O_2^- release in a dose-dependent manner.

Oxidase mobilization in response to *A. phagocytophilum* is contact-dependent, as association with neutrophils initially primed host cells for a more robust response to FMLP within the first 60 min of infection. As infection proceeds, however, more oxidase stores are mobilized. This is supported by a dose-dependent decrease in the respiratory burst of infected cells upon secondary activation with FMLP or PMA. Also, cytochrome b_{558} membrane localization increases over the course of invasion, as does its association with Rac. These data stress the importance of why *A. phagocytophilum* requires a means for protecting itself from released ROS.

Exogenous O_2^- is rapidly scavenged by *A. phagocytophilum* in a dose-dependent manner. The precise mechanism by which this occurs is unclear. A common strategy employed by a wide array of bacteria to rapidly detoxify O_2^- is the use of a periplasm-localized SOD (26). Transcription of an *A. phagocytophilum sodB* homolog has been demonstrated in infected HL-60 cells and in the blood of patients with human

anaplasmosis (32). However, *sodB* genes typically encode Fe-cofactored SODs involved in protection from intracellularly-derived oxidative radicals such as those arising as metabolic by-products, while the Cu/Zn-cofactored SodC proteins protect bacteria from extracellular O_2^- (26). An *A. phagocytophilum sodC* homolog has yet to be demonstrated. Furthermore, recombinant SOD added to the xanthine-xanthine oxidase system completely inhibited O_2^- generation, while only partial reduction was observed in the presence of *A. phagocytophilum*, even at high bacterial concentrations. Therefore, *A. phagocytophilum* either expresses SOD at a lowered concentration relative to the amount of recombinant SOD used as an experimental control or it utilizes an undefined, less efficient mechanism for detoxifying exogenous O_2^-.

Still, the efficiency with which the *A. phagocytophilum* O_2^- scavenging mechanism proceeds is likely substantial enough to protect it from the levels of ROS it elicits during binding and invasion. For instance, neutrophils incubated with *Anaplasma* exhibited reduced ROS release upon secondary stimulation. This indicated that exposure to the bacterium stimulated some degree of NADPH oxidase mobilization and O_2^- production. However, no change in DCL was observed over 90 min immediately following addition of *Anaplasma* to neutrophils, which confirms the bacterium is able to successfully scavenge released ROS while invading its target cell. Furthermore, *A. phagocytophilum*, whether alone or pre-incubated with autologous serum, stimulates almost no respiratory burst activity. This is even more evident when compared to ROS levels generated in response to another bacterium, such as *E. coli*, or to phagocytic particles, such as OpZ or *A. phagocytophilum* opsonized with anti-*A. phagocytophilum* serum. Herron and Goodman provided evidence for a SOD-independent mechanism by

proposing that *A. phagocytophilum* expresses an outer membrane-tethered cytochrome c oxidase that accepts electrons from O_2^-, thereby converting it to harmless O_2 (M. J. Herron and J. Goodman, Abstr. Am. Soc. for Rickettsiol.-*Bartonella* as an Emerg. Path. Grp. 2001 Joint Conf., abstr. 50, 2001). Consistent with our observations, the O_2^- detoxification mechanism characterized by Herron and Goodman involves at least one heat-labile protein expressed on the *A. phagocytophilum* surface.

Results obtained from our O_2^- detection studies differ from those presented by Mott *et al*, which suggested that *A. phagocytophilum* abolishes neutrophil NADPH oxidase activity within 30 min (28). Their studies relied primarily on the reduction of cytochrome c, which can be measured spectrophotometerically as an indicator of extracellular O_2^- production. Rather than kinetically recording the change in absorbance following the addition of cytochrome c, they performed a single measurement after 2 h. A likely pitfall of these conditions is that reduced cytochrome c can, with time, be re-oxidized by oxidants such as H_2O_2 (35), which would certainly have been generated by activated neutrophils. Alternatively, if *A. phagocytophilum* does employ a surface-exposed cytochrome c oxidase, this would likely have re-oxidized cytochrome c. Thus, at 2 h it is probable that re-oxidized cytochrome c was assayed, which would have falsely indicated that no O_2^- had been produced. In addition, results from their chemiluminescence studies reported ROS production as a percent change in luminol dependent chemiluminescence. This can be problematic, as it does not take into account the resting state of control neutrophils. In fact, changing the presentation of data of our chemiluminescence studies from RLU to percent change in DCL falsely suggests that *A. phagocytophilum* eliminates neutrophil ROS production (data not shown).

Flow cytometric and confocal microscopic studies, which allow for a true assessment of oxidase co-localization, confirmed that neutrophil exposure to *A. phagocytophilum* results in oxidase assembly and cellular activation. It has been suggested that inhibition of neutrophil O_2^- production following *A. phagocytophilum* infection is due to the bacterium actively degrading $p22^{phox}$ (29). Loss of $p22^{phox}$ would prohibit proper formation of the cytochrome b_{558} heterodimer (34), which would effectively prevent O_2^- formation. This hypothesis, however, is not supported by results obtained from these studies. Confocal analysis demonstrated considerably increased $p22^{phox}$ staining of *A. phagocytophilum* infected cells. Western analyses confirmed that $p22^{phox}$ levels are not reduced during *A. phagocytophilum* infection (data not shown). Moreover, surface recognition by mAb 7D5, which binds to $gp91_{phox}$ only when complexed with $p22^{phox}$, directly increases with *A. phagocytophilum* burden. This confirms high levels of $p22^{phox}$ and more importantly demonstrates assembly of functional cytochrome b_{558} in response to A. *phagocytophilum* infection.

Following neutrophil invasion, *A. phagocytophilum* resides within a vacuole that fails to label with antibodies against $gp91^{phox}$ and $p22^{phox}$, despite its immediate proximity to cytochrome b_{558}-carrying secretory vesicles. This suggests the vacuolar membrane excludes NADPH oxidase and thereby protects the bacterium from intracellularly directed ROS. The *A. phagocytophilum* vacuole is a privileged site indeed, as it also fails to accumulate late endosomal markers and prevents luminal acidification and phagolysomal fusion (27, 38). Excluding the phagocyte NADPH oxidase from its parasitophorous vacuole may be a common virulence theme among intracellular bacteria, as this was first identified as an intra-macrophage survival strategy of *Salmonella*

typhimurium. The ability of *S. typhimurium* to exclude $p22^{phox}$ and $p47^{phox}$ (36), as well as vesicles containing inducible nitric oxide synthase (9), from its vacuole is conferred by the presence of a type III secretion system encoded by *Salmonella* pathogenicity island II. *A. phagocytophilum*, however, is the only pathogen for which exclusion of neutrophil NADPH oxidase from the parasitophorous vacuole has been demonstrated. The means by which it does so is not known.

A. phagocytophilum's tropism for neutrophils makes it highly unique among microorganisms and indicates that it effectively protects itself from the oxidative killing capacity of its host cell. The data presented here demonstrate that *Anaplasma* entry into its host neutrophil is a prolonged process, during which it stimulates NADPH oxidase assembly and plasma membrane redistribution. This indicates the bacterium would be exposed to O_2^- upon binding and invasion. Based on our data, we hypothesize that *A. phagocytophilum* is able to protect itself by directly detoxifying O_2^- in its local environment using a currently undefined mechanism. Additionally, following neutrophil invasion, *A. phagocytophilum* resides within a protective vacuole that excludes NADPH oxidase. Future studies will seek to define the molecular means by which *A. phagocytophilum* directly scavenges O_2^- and prevents fusion of oxidase carrying vesicles with its protective parasitophorous compartment. Such efforts will provide a thorough understanding of how this inimitable bacterium combats the neutrophil respiratory burst.

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Chapter 4

Rac2 is critical for neutrophil primary granule exocytosis

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Figure 4.1 C was contributed by Ms. Melissa Steward

Figure 4.5 C was contributed by Ms. Melissa Steward and Dr. Dawn L. Macdonald

Abstract

Neutrophil degranulation is important in many inflammatory disorders, although the intracellular mechanisms underlying this process remain poorly understood. The Rho GTPase, Rac2, has been implicated in control of degranulation in earlier studies. We hypothesized that Rac2 selectively regulates neutrophil primary granule release. Using bone marrow and peritoneal exudate neutrophils from *rac2*$^{-/-}$ mice in comparison with similar cells from wild type C57Bl/6 mice, we found that primary granule myeloperoxidase and elastase release was absent in Rac2$^{-/-}$ neutrophils in response to chemoattractant stimulation, cytochalasin B/f-Met-Leu-Phe (CB/fMLP) and CB/leukotriene B_4. Rac2$^{-/-}$ neutrophils also failed to exhibit mobilization of the primary granule marker CD63^{+} during CB/fMLP stimulation as determined by confocal microscopy. Priming of Rac2$^{-/-}$ neutrophils with TNF or by peritoneal elicitation did not rescue the defect in primary granule release. However, phosphorylation of p38 MAP kinase in Rac2$^{-/-}$ neutrophils was evident in response to CB/fMLP and/or TNF. Primary granule density and morphology were normal in Rac2$^{-/-}$ neutrophils. Secondary specific and tertiary granule release, measured by lactoferrin immunoassay and zymography, was normal in response to CB/fMLP and adhesion to fibronectin. These findings suggest an obligatory role for Rac2 in regulation of primary granule release by neutrophils.

Introduction

Neutrophils constitute 40-80% of circulating white blood cells and are a critical component in the maintenance of innate immunity. Overexuberant activation of neutrophils can lead to extensive degranulation, which may be fatal in septic shock, acute lung injury, and other serious inflammatory disorders. Neutrophils contain four different granule populations; primary azurophilic, secondary specific, tertiary granules, and secretory vesicles. These granule types exhibit a hierarchy of release in response to intracellular Ca^{2+} spikes,[1] suggesting that granule-specific pathways exist to regulate their secretion. Earlier studies using permeabilized or patch-clamped neutrophils have shown that the final step of granule fusion with membrane, leading to release of granule contents, is dependent on guanosine triphosphate (GTP) and Ca^{2+}.[2,3] Determining the identity of GTPase(s) involved in this step has been elusive. Recently, Rho GTPases Rac1, Rac2, and Cdc42 have been suggested as possible exocytotic GTPases.[4]

Rho GTPases, an 18-member subfamily of *ras*-related GTPases, have been shown to be important in regulating a diverse array of cell activation events downstream of receptor activation.[5] Their principle functions are associated with regulation of cytoskeletal reorganization, formation of lamellipodia, chemotaxis, transcriptional events, and cell growth and differentiation.[6] Rac1 and Rac2 exhibit distinct tissue distribution patterns; while Rac1 is ubiquitously expressed, Rac2 is limited to hematopoietic cells.[7] Human neutrophils primarily express Rac2 rather than Rac1,[8] while murine neutrophils express equivalent levels of Rac1 and Rac2.[9] Although Rho GTPases are involved in regulating cell functions at different levels, each isoform demonstrates substantial cross-reactivity with each other. Rac1 and Rac2 exhibit considerable homology at the amino

acid level (92% identity)[10] and are functionally interchangeable in their ability to activate cytoskeletal remodeling in chemotaxis, as well as superoxide generation through the respiratory burst NADPH oxidase complex.[11] Such functional overlap may be partially due to the ability of Rac1 and Rac2 to activate common downstream effectors, such as those containing CRIB motifs (p21-activating kinases 1-6, among others), and in cascades associated with c-Jun kinase and p38 MAP kinase.[12,13] Inhibition of p38 MAP kinase with SB203580 has been demonstrated to partially block f-Met-Leu-Phe (fMLP)-induced primary and secondary granule release in neutrophils, suggesting an important role for p38 MAP kinase in activating receptor-mediated degranulation.[14]

Rac2 and Cdc42 have been implicated in the regulation of degranulation in mast cells.[4,15,16] Addition of active recombinant isoforms of Rac2 and Cdc42 delayed the loss of sensitivity to GTPγS and Ca^{2+} during rundown of exocytotic responses in permeabilized rat mast cells,[4] while dominant-negative forms of Cdc42 and Rac1 introduced by vaccinia virus vectors into RBL-2H3 cells inhibited antigen-induced secretion.[16] In addition, *Clostridium difficile* toxin B, an inhibitor of Rac1, Rac2 and Cdc42 but not RhoA, blocked FcεRI-mediated degranulation from RBL cells.[17,18] However, these studies could not discriminate between pathways selectively regulated by distinct GTPase isoforms. Thus, the use of gene deletion models is essential for defining the role of each specific Rho GTPase isoform in degranulation.

Gene deletion of Rac2 in mice was shown to lead to a profound loss of chemotactic ability in peripheral blood and bone marrow neutrophils, as well as decreased superoxide production in response to fMLP, tumor necrosis factor (TNF) or phorbol myristate acetate (PMA).[19] Phosphorylation of p38 MAP kinase was slightly

decreased in stimulated Rac2$^{-/-}$ neutrophils,[20] while that of ERK1/2 was more significantly diminished.[19]

Recently, a male infant was reported expressing a dominant negative form of Rac2 (Rac2D57N),[21,22] which led to multiple life-threatening infections. Peripheral blood neutrophils from this child exhibited marked deficiencies in adhesion and respiratory burst, while degranulation assays suggested a defect in primary granule release. However, degranulation responses have not been studied in detail in Rac2-deficient neutrophils.

In this study, we hypothesized that Rac2 is a crucial regulator of neutrophil primary granule release. Peritoneal exudate and bone marrow neutrophils from *rac2*$^{-/-}$ mice were harvested to investigate degranulation responses to chemoattractant stimulation. We show an absence of primary granule release, with intact secondary and tertiary granule exocytosis, in Rac2$^{-/-}$ neutrophils.

Methods

Materials. Chemicals were purchased from Sigma-Aldrich Co. (Oakville, ON) unless otherwise noted. Ca^{2+}, Mg^{2+}, and phenol red-free Hank's balanced salt solution (HBSS) and phosphate-buffered saline (PBS, pH 7.2) were obtained from Life Technologies (Grand Island, NY). HBSS (pH 7.4) was supplemented with BSA (0.1%) and glucose (1%) to make HBSS-BG. Phenol red-free RPMI-1640 was purchased from Life Technologies. Recombinant murine TNF was from BD Pharmingen (San Diego, CA). Antibodies to p38 MAP kinase and phospho-p38 were obtained from BD Biosciences (Mississauga, ON).

Animals. The *rac2*$^{-/-}$ mice used in this study were previously generated by targeted disruption of the *rac2* gene,[19] which had been backcrossed into C57Bl/6 mice for > 11 generations. Wild-type C57Bl/6 control mice were from Charles River Canada, Saint-Constant, PQ. Animals were housed under specific pathogen-free conditions and fed autoclaved food and water as needed. Mice used in these experiments were between 8 – 20 wk of age.

Preparation of bone marrow and peritoneal neutrophils. Bone marrow neutrophils (BMN) were obtained by density gradient centrifugation using a modification of previously described techniques.[20,23] Briefly, BMN were isolated from femurs and tibias flushed with 3 ml of HBSS-BG using a 22-gauge needle (Becton Dickinson, San Jose, CA). Cells were pelleted by centrifugation at 300*g* for 10 min at 4°C before mixing with 45% Percoll (Pharmacia, Uppsala, Sweden) and layering onto gradients consisting of 3 ml 81%, 2 ml 62%, 2 ml 55%, and 2 ml 50% Percoll in HBSS-BG. After centrifugation of gradients at 1600*g* for 30 min at 10°C, BMN were removed from the

interface between the 62% and 81% layers and washed twice with HBSS-BG. To remove contaminating red blood cells, BMN were centrifuged on Histopaque 1119 at 1600*g* for 30 min at 10°C, and washed twice in HBSS-BG. We obtained 66-70% neutrophils for WT and $Rac2^{-/-}$ bone marrow preparations as determined by Diff-Quik staining, which is similar to previously published values.[20] Neutrophil viability was >90% as determined by trypan blue exclusion.

Peritoneal exudate neutrophils (PEN) were isolated by intraperitoneal injection of mice with 2 ml 0.05% sodium caseinate in sterile saline 4 h prior to sacrifice and peritoneal lavage with 6-8 ml HBSS-BG supplemented with 2 U/ml heparin.[24] Cells were washed with phenol red-free RPMI-1640 and stained with Kimura's stain for differential counts. The purity averaged between 88-94% for neutrophils from nine animals, with contaminating mast cells. Injection of animals with sodium caseinate was essential for eliciting PEN, since no PEN could be obtained from uninjected animals.

Incubation of cells for degranulation assays. PEN and BMN were washed and resuspended at 5-10 x 10^6 cells/ml in phenol red-free RPMI-1640 for all degranulation assays, which were carried out on cells in suspension. Aliquots (50 µl) of cells were preincubated with 5 µg/ml cytochalasin B (CB) for 5 min at 37°C before adding increasing doses of fMLP (0-40 µM) or leukotriene B_4 (LTB_4, 0-1 µM). Cells were incubated for a further 15 min (for fMLP) or 1 h (for LTB_4) before terminating by centrifugation at 4°C for 5 min at 300*g*. Supernatants were removed for assay of released granule proteins, while unstimulated cells were lysed by addition of an equivalent volume of RPMI-1640 containing 0.5% CHAPS. For fibronectin adhesion assays, BMN were incubated on recombinant human fibronectin-coated plates (coated with RetroNectin

from PanVera, Fisher Scientific, Nepean, ON) for 1 h at 37°C in a humidified incubator as previously described,[25,26] and in some experiments, stimulated for a further 15 min with 5 μM fMLP.

Measurement of myeloperoxidase, elastase, and lactoferrin. Myeloperoxidase (MPO), a marker for primary granules, was assayed by using tetramethylbenzidine (TMB) based on a previously established technique.[27] Briefly, 150 μl of TMB substrate solution was added to 50 μl of sample and incubated (ambient temperature, 30 min) prior to termination with 50 μl 1 M H_2SO_4. Plates were read spectrophotometrically at 450 nm (Molecular Devices, Sunnyvale, CA). Elastase activity was measured by an EnzCheck Elastase assay kit according to manufacturer's instructions (Molecular Probes, Eugene, OR). Briefly, 100 μl fresh sample was incubated with 100 μl substrate solution (DQ elastin labelled with BODIPY FL dye) for 24 h at room temperature in the dark (shorter incubations generated less fluorescence) before reading the resulting fluorescence at 505 nm for excitation and 515 nm for emission. Absorbance values for released MPO and elastase were divided into the average of values from 0.5% CHAPS-lysed cells to give percentage of total cellular mediator released.

Lactoferrin (LTF) was assayed by ELISA measurement based on a previously published observation demonstrating cross-reactivity of human anti-LTF (Sigma-Aldrich) for murine LTF.[28] Values of release were plotted as optical density, since this assay technique produced low values for total cellular LTF.

Zymography. Supernatants of resting and stimulated cells were subjected to gel electrophoresis and analyzed for gelatinase activity as previously described.[29] Briefly, 4X zymography loading buffer (40% glycerol, 8% SDS, 20 μg/ml bromophenol blue in 0.25

M Tris, pH 6.8) was added to cell supernatants and the mixture was loaded on 10% acrylamide ready-made zymogram gels (BioRad Laboratories, Hercules, CA). Electrophoresis was carried out at 160 mV for 2 h and gels were washed 3 times for 20 min with 2.5% Triton X-100 at room temperature with shaking. Gels were incubated with zymography buffer (0.9% NaCl, 5 μM $CaCl_2$, 0.00025% NaN_3 in 50 mM Tris) for up to 1 week at 37°C. Gel staining was carried out using 0.5% Coomassie blue G-250 in 25% methanol, 10% acetic acid for 1 h with shaking at room temperature, followed by destaining overnight with 4% methanol and 8% acetic acid.

Confocal microscopy. Confocal analysis of neutrophil cytospins was carried out as previously reported [30] using 10 μg/ml mouse monoclonal antibody to CD63 (Serotec Inc., Raleigh, NC). Binding of CD63 was detected with 2 μg/ml goat anti-mouse IgG secondary antibody conjugated to Alexa 488 (Molecular Probes, Eugene, OR).

Electron microscopic analysis of BMN. BMN were pelleted from WT and *rac2*$^{-/-}$ mice and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer before treatment with diaminobenzidine (DAB) solution for 1 h (0.01% H_2O_2, 0.05 M Tris-HCl, pH 6.8, and 5 mg/ml DAB), using a modification of a previously described technique,[31] to enhance staining of peroxidase-containing granules. Samples were submitted to conventional fixation procedure with 1% osmium tetroxide and sequential dehydration steps before addition of Epon resin and sectioning onto uncoated grids. Sections were subjected to a postfix staining step with lead citrate and uranyl acetate, then analysed (Hitachi model H7000 transmission electron microscope).

Data presentation. Release of MPO and elastase were calculated as a percentage of total cellular mediator activity by dividing the corrected absorbance of supernatants into the

sum of supernatants and average corrected values for lysed pellets. Data was analyzed by two-tailed Student's *t*-test or one-way ANOVA analysis followed by Tukey's column comparison, and depicted in figures as mean ± SEM. All figures shown represent averages of at least three separate experiments.

Results

Rac2 is essential for primary granule release in response to chemoattractants.

Stimulation of neutrophils *in vitro* with cytochalasin B in combination with chemoattractants results in release of primary granule contents, which include mediators that are crucial for microbial killing.[32] We tested the effect of CB/fMLP on primary granule release from freshly prepared WT and $Rac2^{-/-}$ BMN by measuring the release of MPO. As shown in Figure 4.1A, MPO release from WT neutrophils was detected in response to 5-40 μM fMLP stimulation, with maximal release at 5 μM fMLP (31 ± 2%). In contrast, $Rac2^{-/-}$ BMN were defective in their ability to release primary granule MPO during CB/fMLP stimulation. Maximal MPO release from $Rac2^{-/-}$ neutrophils was 4.4 ± 0.5% (5 μM fMLP), which did not significantly differ from baseline values obtained in unstimulated $Rac2^{-/-}$ BMN. WT and $Rac2^{-/-}$ BMN treated with CB alone did not release significant levels of MPO (2.6 ± 1.9% and 0.8 ± 0.8%, respectively). The MPO content in lysed unstimulated cells was not significantly different between WT and $Rac2^{-/-}$ BMN (cell pellets averaged 0.34 ± 0.04 vs. 0.46 ± 0.23 OD, respectively), suggesting that MPO was synthesized and stored at similar levels in $Rac2^{-/-}$ BMN to those of WT BMN. Similar release profiles were obtained with BMN stimulated by dihydrocytochalasin B (5 μg/ml) and 0.1-40 μM fMLP (data not shown), suggesting that preincubation with CB did not adversely affect secretion because of interference with glucose transport. Similarly, increasing doses of another chemoattractant, LTB_4, induced the release of MPO from WT BMN but not $Rac2^{-/-}$ BMN (Figure 4.1B). Addition of 50 U/ml superoxide dismutase did not enhance CB/fMLP-induced secretion of MPO, suggesting that released MPO was not significantly inactivated by MPO-catalyzed oxidation.[33]

These findings suggest that $Rac2^{-/-}$ neutrophils exhibit a deficiency in MPO release in response to stimulation by two different agonists.

We were unable to obtain significant MPO release in WT or $Rac2^{-/-}$ BMN in response to murine recombinant TNF (50 ng/ml, 5 min) and fMLP (0-40 μM, 15 min) (data not shown). Fibronectin adhesion (1 h) induced insignificant MPO release from WT (5 ± 2%) and $Rac2^{-/-}$ (0.2 ± 0.15%) BMN. Addition of fMLP (0-40 μM) to fibronectin-adhered BMN did not enhance MPO release in WT (4.8 ± 0.5%) or $Rac2^{-/-}$ (0.8 ± 0.75%) BMN. This is in contrast to human neutrophils which release elastase in response to fibronectin.[34] These findings suggest that cytochalasin B pre-treatment is necessary to induce primary granule release from neutrophils, as supported by previous studies.[32,35] We also tested the effect of two Ca^{2+} ionophores, A23187 and ionomycin, on MPO release from BMN, but these failed to induce significant release from WT or $Rac2^{-/-}$ cells at 1-10 μM (data not shown). The inability of murine BMN to release MPO in response to these stimuli may indicate a species difference, since human neutrophils release primary granule products in response to similar conditions (data not shown).[32,34,36]

In order to confirm that primary granule mediator release was deficient in $Rac2^{-/-}$ BMN, we measured elastase activity as an additional primary granule marker. Elastase release was also abolished in CB/fMLP-stimulated $Rac2^{-/-}$ BMN compared with WT cells (Figure 4.1C). Similar results were obtained in cells stimulated with CB/LTB_4 (data not shown). These observations suggest that Rac2 gene deletion leads to loss of primary granule mediator release in neutrophils.

The responsiveness of $Rac2^{-/-}$ neutrophils to respiratory burst stimulation was confirmed by testing BMN from *rac2*$^{-/-}$ and WT mice using a conventional cytochrome *c*

reduction assay. BMN from Rac2$^{-/-}$ mice generated 53% less O_2^- than WT controls in response to stimulation with 500 ng/ml PMA (0.51 ± 0.07 compared with 1.1 ± 0.2 nmol $O_2^-/10^6$ cells/min, respectively, $p < 0.05$). These values correlate with those of previously published data,[19] and suggest that these animals are genetically and phenotypically similar to those previously studied.

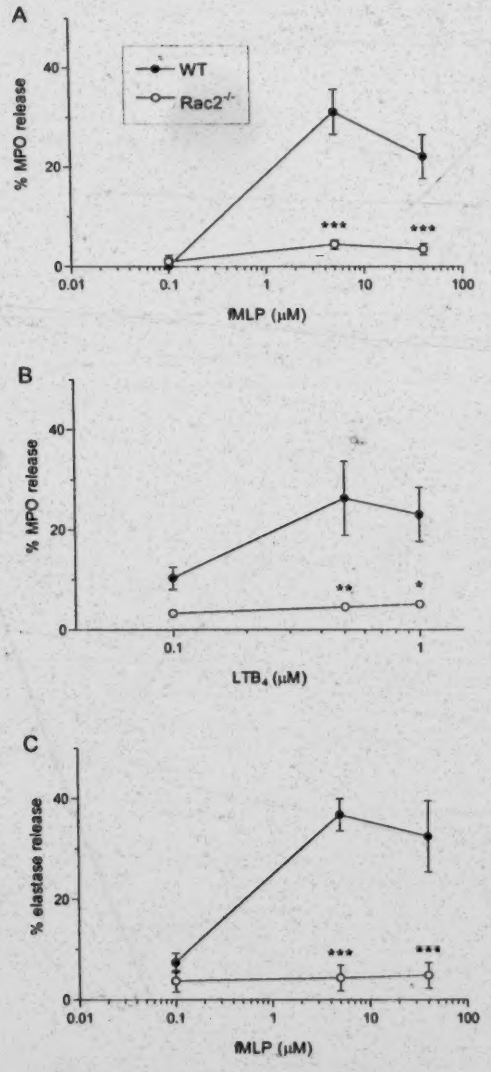


Figure 4.1. Absence of primary granule release in chemoattractant-stimulated Rac2$^{-/-}$ neutrophils.

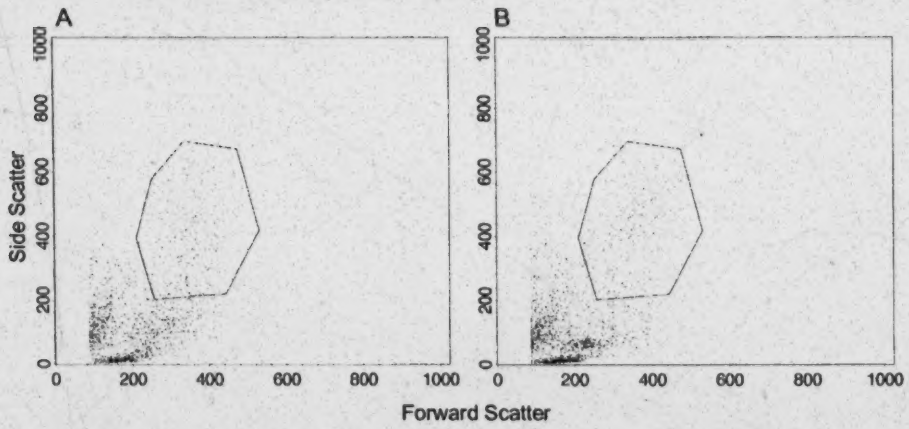
Freshly prepared BMN from WT and *rac2*$^{-/-}$ neutrophils were examined for their ability to release the primary granule marker MPO in response to increasing doses of fMLP (A) for 15 min following 5 min preincubation with 5 μg/ml CB. MPO release was determined and shown as percentage of total cellular lysate values. (B) Neutrophils were stimulated with increasing doses of LTB_4 for 1 h following 5 min incubation with 5 μg/ml CB. (C) Elastase activity in supernatants of neutrophils stimulated with CB/fMLP. * indicates $p < 0.05$, ** $p < 0.02$, and *** $p < 0.01$. (Figure 4.1 was generated by D. Abdel Latif (A, B) and M. Steward (C))

CD63 translocation in Rac2$^{-/-}$ neutrophils is deficient in response to CB/fMLP. To determine if primary granules translocate to the membrane in response to CB/fMLP in Rac2$^{-/-}$ neutrophils, cytospins of resting and stimulated WT and Rac2$^{-/-}$ BMN were prepared and labeled for CD63 immunoreactivity. Resting WT and Rac2$^{-/-}$ BMN exhibited a homogeneous cytoplasmic staining, suggesting the presence of primary granules in the cytoplasm (Figure 4.2A & B). Following stimulation with CB/fMLP (5 μM, 15 min) of WT BMN, large aggregates of CD63 immunoreactivity formed in submembranous regions, suggesting that CD63^{+} granules had translocated to the cell membrane (Figure 4.2C). In contrast, Rac2$^{-/-}$ BMN did not display any change in CD63 labeling from that of resting cells (Figure 4.2D), suggesting that granule trafficking may be deficient in Rac2$^{-/-}$ neutrophils. It was not possible to resolve the structure of individual CD63^{+} granules by confocal analysis, as these are smaller than the limit of resolution of this technique (~0.3 μm in diameter).[3]

Granular morphology in WT and Rac2$^{-/-}$ neutrophils is identical. Changes in granule morphology in Rac2$^{-/-}$ neutrophils may explain the defect in primary granule release, such as an overall reduction in granule volume or numbers. To test whether neutrophil morphology from *rac2$^{-/-}$* mice was altered in comparison with WT cells, BMN were subjected to analysis by flow cytometry and assessed for their size and granularity. There were no significant differences in mean forward and side scatter values obtained from gated BMN from WT and *rac2$^{-/-}$* mice (Figure 4.3), suggesting that cell sizes and granularity of BMN were similar. BMN from WT and *rac2$^{-/-}$* animals were further analyzed by electron microscopy using a conventional osmium tetroxide-based fixation technique coupled with DAB staining to enhance the electron density of peroxidase-

containing organelles. Both WT and Rac2$^{-/-}$ neutrophils exhibited identical granule morphology in BMN (Figure 4.4), with no apparent difference in granule volume, numbers, or electron density. The similarity in cellular morphology was evident in all cells observed. These findings suggest that *rac2* gene deletion did not affect granulogenesis in BMN, or any other apparent aspects of cellular morphology in resting cells.

Figure 4.2. CD63^{+} granule translocation is inhibited in Rac2$^{-/-}$ neutrophils. Cytospins were prepared of freshly isolated BMN treated with CB/fMLP (5 μM) for 15 min, stained with 10 μg/ml anti-CD63 as a primary granule marker (green) and counterstained with DAPI (blue) to show nuclear structure. Panels show representative images from (A) resting WT BMN, (B) resting Rac2$^{-/-}$ BMN, (C) CB/fMLP-stimulated WT BMN, and (D) CB/fMLP-stimulated Rac2$^{-/-}$ BMN, with the left panel in each case showing CD63 alone and right panels showing combined images of CD63 immunoreactivity and DAPI nuclear stain. Original magnification, 40X. (Figure 4.2 was generated by D. Abdel Latif)



Cell type	Gated events	FS Mean	SS Mean
A) WT BMN	2279	347.04	417.47
B) Rac2$^{-/-}$ BMN	2570	349.37	404.11

Figure 4.3. Similar granularity in WT and Rac2$^{-/-}$ neutrophils.
BMN were isolated from (A) WT and (B) Rac2$^{-/-}$ mice and subjected to flow cytometric analysis to analyze their side scatter properties. Each scatter plot shows 10,000 events. Gates in the scatter plots are arbitrarily selected to show representative means of forward scatter (FS) and side scatter (SS). (Figure 4.3 was generated by D. Abdel Latif)

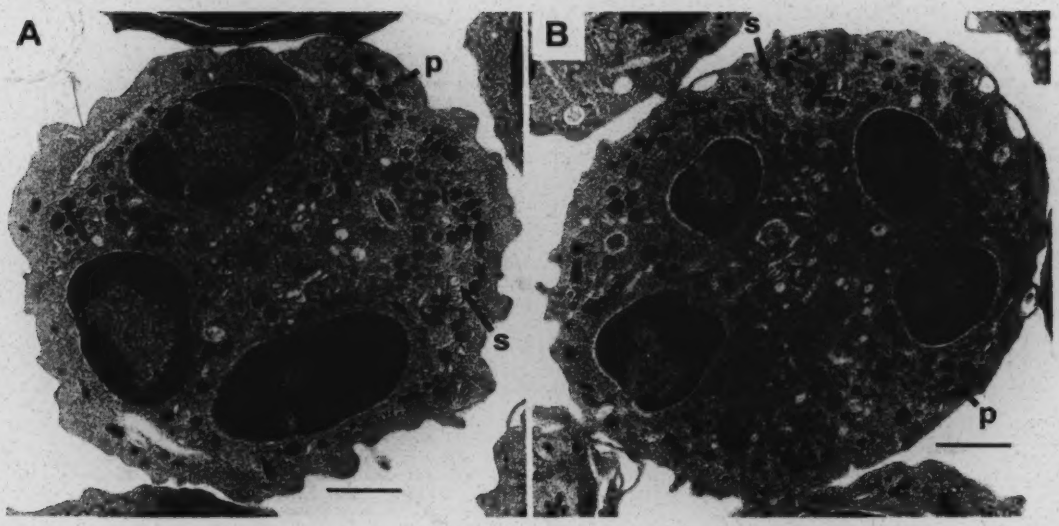


Figure 4.4. Normal granular morphology in neutrophils from *rac2*$^{-/-}$ mice. Neutrophils were pelleted from bone marrow and sectioned for EM analysis. Panels show typical cells from (A) WT and (B) *rac2*$^{-/-}$ mice. Labels indicate p = primary granule, s = secondary granule, G = Golgi apparatus. The *bar* indicates 1 μm. (Figure 4.4 was generated by D. Abdel Latif)

Loss of fMLP-induced primary granule release in Rac2$^{-/-}$ neutrophils is not rescued by in vitro *priming with TNF.* In order to determine if *in vitro* priming may reconstitute the release of primary granule release in Rac2$^{-/-}$ neutrophils, TNF was preincubated with cells prior to their activation with CB/fMLP. Pre-treatment of Rac2$^{-/-}$ BMN with TNF (50 ng/ml) failed to correct the defect in MPO release following CB/fMLP stimulation (Figure 4.5A). Separate experiments were done to confirm that Rac2$^{-/-}$ neutrophils were responsive to TNF stimulation by carrying out flow cytometric analysis for surface expression of adhesion markers CD11b and CD18. In Rac2$^{-/-}$ neutrophils stimulated by TNF at 50 ng/ml for 10 min, CD11b and CD18 were upregulated to a similar degree as that seen in WT neutrophils, indicating that cells were effectively primed by TNF incubation (data not shown).

p38 MAP kinase phosphorylation in Rac2$^{-/-}$ neutrophils is induced by TNF and CB/fMLP. TNF is a potent stimulus for p38 MAP kinase phosphorylation in human neutrophils, and may be required for fMLP-induced primary and secondary granule release as well as IL-8 secretion.[14,37] We determined whether TNF might also activate p38 kinase in murine BMN in order to prime these cells for degranulation. Phosphorylation of p38 was investigated in WT and Rac2$^{-/-}$ BMN before and after stimulation with TNF (50 ng/ml, 5 min), CB/fMLP, and TNF plus CB/fMLP (Figure 4.5B). In WT neutrophils, TNF and CB/fMLP induced p38 phosphorylation, although TNF was less potent in this regard. This is in contrast to human neutrophils, where TNF was shown to be a potent inducer of p38 phosphorylation.[37] Addition of TNF to CB/fMLP did not enhance phosphorylation in response to CB/fMLP. Interestingly, Rac2$^{-/-}$ neutrophils exhibited nearly normal levels of p38 phosphorylation in response to

TNF and/or CB/fMLP compared with WT cells. These findings suggest that p38 MAP kinase activation alone is not sufficient for triggering primary granule exocytosis.

Defect in fMLP-induced primary granule release is not due to an in vivo *priming deficiency in Rac2$^{-/-}$ neutrophils. In vivo* priming of neutrophils through peritoneal elicitation is likely to be a stronger stimulus for cell priming than *in vitro* treatment with cytokines. In peritoneal lavage cells, WT neutrophils exhibited a significant dose-dependent release of primary granule MPO in response to CB/fMLP, with responses reaching maximal values at 5 μM fMLP (78 ± 4% of total cellular MPO), as shown in Figure 4.5C. In contrast, PEN from *rac2$^{-/-}$* mice demonstrated a lack of MPO release under similar stimulation conditions (Figure 4.5C). Rac2$^{-/-}$ PEN showed no increase above baseline levels of spontaneous MPO release (14 ± 2%) in response to all concentrations of fMLP tested in this study. MPO release from WT PEN was considerably greater than that of WT BMN, presumably due to their primed state. These observations rule out the possibility that the loss of primary granule release may be due to a priming defect in Rac2$^{-/-}$ neutrophils.

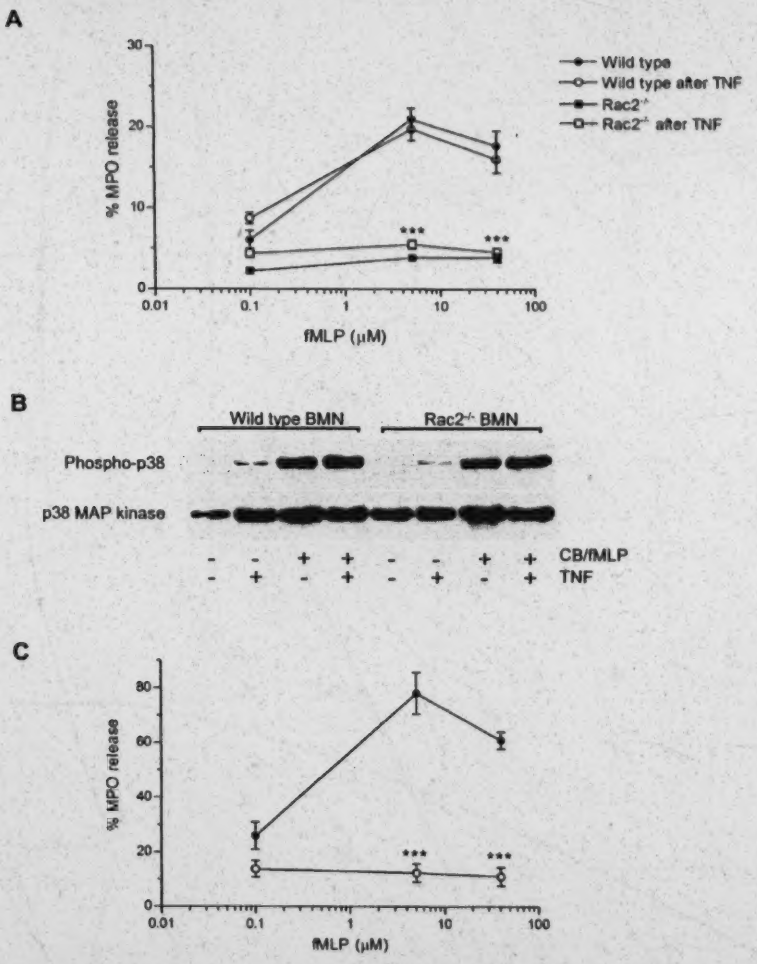


Figure 4.5. Priming of Rac2$^{-/-}$ neutrophils fails to reverse the degranulation defect. WT and Rac2$^{-/-}$ BMN were stimulated with CB/fMLP (5 μM) with or without preincubation of 50 ng/ml TNF for 5 min. (A) MPO release from WT and Rac2$^{-/-}$ BMN in the presence or absence of TNF preincubation. *** indicates $p < 0.01$ compared with TNF-preincubated WT controls at the same doses of fMLP. (B) Phosphorylation of p38 MAP kinase in Rac2$^{-/-}$ neutrophils is normal in response to TNF and CB/fMLP. Western blot analysis was carried out for phospho-p38 and p38 MAP kinase in cellular lysates. (C) PEN were treated with increasing doses of fMLP for 15 min following 5 min preincubation with 5 μg/ml CB. MPO release was determined and shown as percentage of total cellular lysate values. *** indicates $p < 0.01$. (Figure 4.5 was generated by D. Abdel Latif (A, B), M. Steward and D. Macdonald (C))

Secondary granule release is normal in Rac2$^{-/-}$ neutrophils. We next tested whether the loss of Rac2 function might also affect the release of another important granule population in neutrophils, secondary granules, which release bacteriostatic mediators such as LTF during phagocytosis and degranulation. The release of LTF from BMN was measured in response to stimulation with CB/fMLP. There was normal release of LTF from Rac2$^{-/-}$ BMN in response to CB/fMLP stimulation (Figure 4.6A), which was equivalent to that of WT neutrophils. However, treatment of WT and Rac2$^{-/-}$ BMN with CB alone resulted in significant release of LTF (0.23 ± 0.04 OD for WT and Rac2$^{-/-}$ cells, after subtracting background of untreated resting cells). This represented between 50-67% of the total releasable LTF in response to maximal stimulation (40 μM fMLP). To determine the release of LTF under more physiological conditions, freshly prepared BMN were adhered to fibronectin-coated plates for 1 h and their supernatants assayed for LTF release. Both WT and Rac2$^{-/-}$ BMN released LTF following adhesion, representing approximately 24-26% of the total releasable LTF (Figure 4.6B). This release was not significantly enhanced by addition of increasing doses of fMLP (0-40 μM) or co-incubation with TNF (10 ng/ml). Addition of 500 ng/ml PMA induced ~59-81% of total releasable LTF, which did not differ in WT or Rac2$^{-/-}$ BMN, suggesting that LTF secretion in response to protein kinase C (PKC) activation is similar in both WT and Rac2$^{-/-}$ BMN. In our hands, this assay was relatively insensitive for detecting LTF release. However, we were able to determine that there was no significant difference in LTF release in WT and Rac2$^{-/-}$ BMN, indicating that this pathway of exocytosis is independent of Rac2.

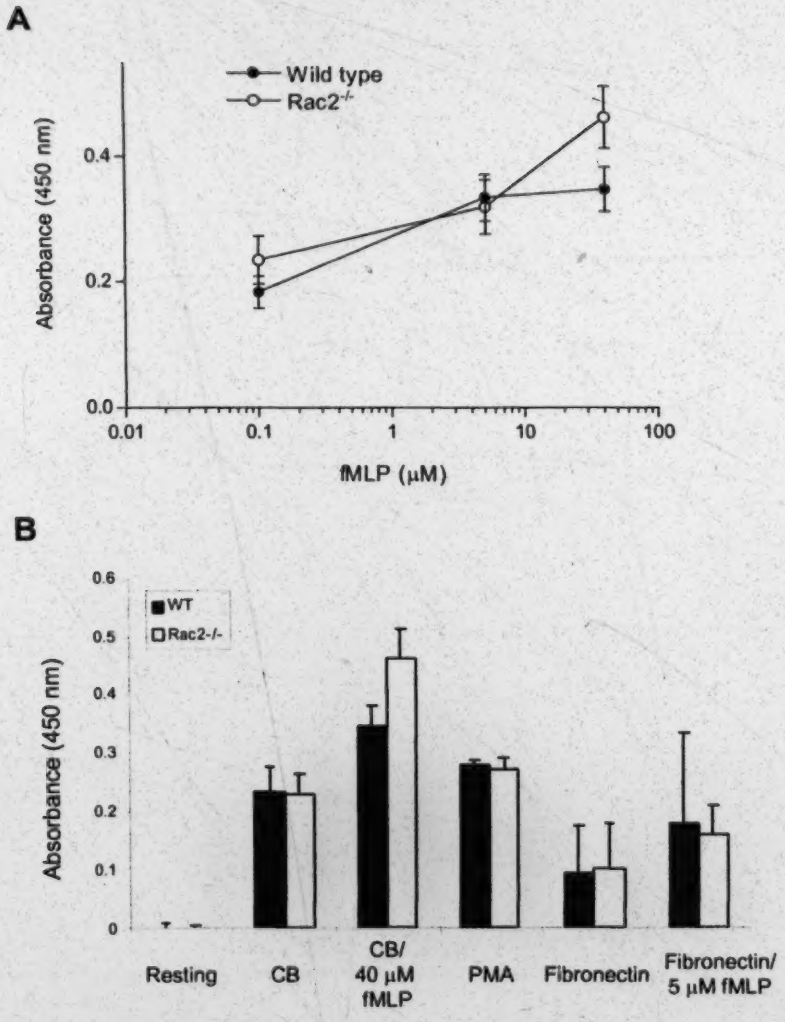


Figure 4.6. Secondary granule LTF release is equivalent in WT and Rac2$^{-/-}$ neutrophils.
BMN were stimulated with increasing doses of (A) fMLP (after CB preincubation). Release was significant ($p < 0.05$) at the highest dose of agonist. (B) Comparison of the effects of CB, CB/40 μM fMLP, PMA (500 ng/ml), fibronectin adhesion (1 h), or fibronectin (1 h) followed by 5 μM fMLP on LTF release determined by immunoassay. (Figure 4.6 was generated by D. Abdel Latif)

Tertiary granule release is not affected in Rac2$^{-/-}$ neutrophils. Supernatants of CB/fMLP-stimulated WT and Rac2$^{-/-}$ BMN were subjected to zymography analysis to determine the release of gelatinase. Unstimulated cells released negligible quantities of gelatinase activity, while cells stimulated with CB alone or CB plus 5 μM fMLP released a significant amount of gelatinase activity migrating at ~105 kDa (Figure 4.7A). This molecular weight corresponds with the expected size of murine matrix metalloprotease-9 (MMP-9),[38] a marker for the tertiary granules in neutrophils.[1] Stimulation of BMN with CB/fMLP did not enhance the release of MMP-9 over CB alone (Figure 4.7). Total cell MMP-9 was equivalent in WT and Rac2$^{-/-}$ BMN based on densitometric analysis of zymography bands from cell lysates, suggesting that Rac2$^{-/-}$ BMN were not deficient in MMP-9 synthesis and storage (data not shown). Similarly to LTF release, CB alone induced significant gelatinase release in BMN from WT and *rac2*$^{-/-}$ mice (78-80% of total releasable MMP-9). However, we found that MMP-9 release occurred under physiological conditions in WT and Rac2$^{-/-}$ BMN in response to adhesion to fibronectin alone (1 h) and fibronectin (1 h) plus 5 μM fMLP (Figure 4.7). Addition of fMLP enhanced the release of MMP-9 when added to fibronectin-adhering cells. Cells released between 22-32% of maximal MMP-9 release in response to fibronectin adhesion followed by fMLP (5 μM) stimulation for 15 min.

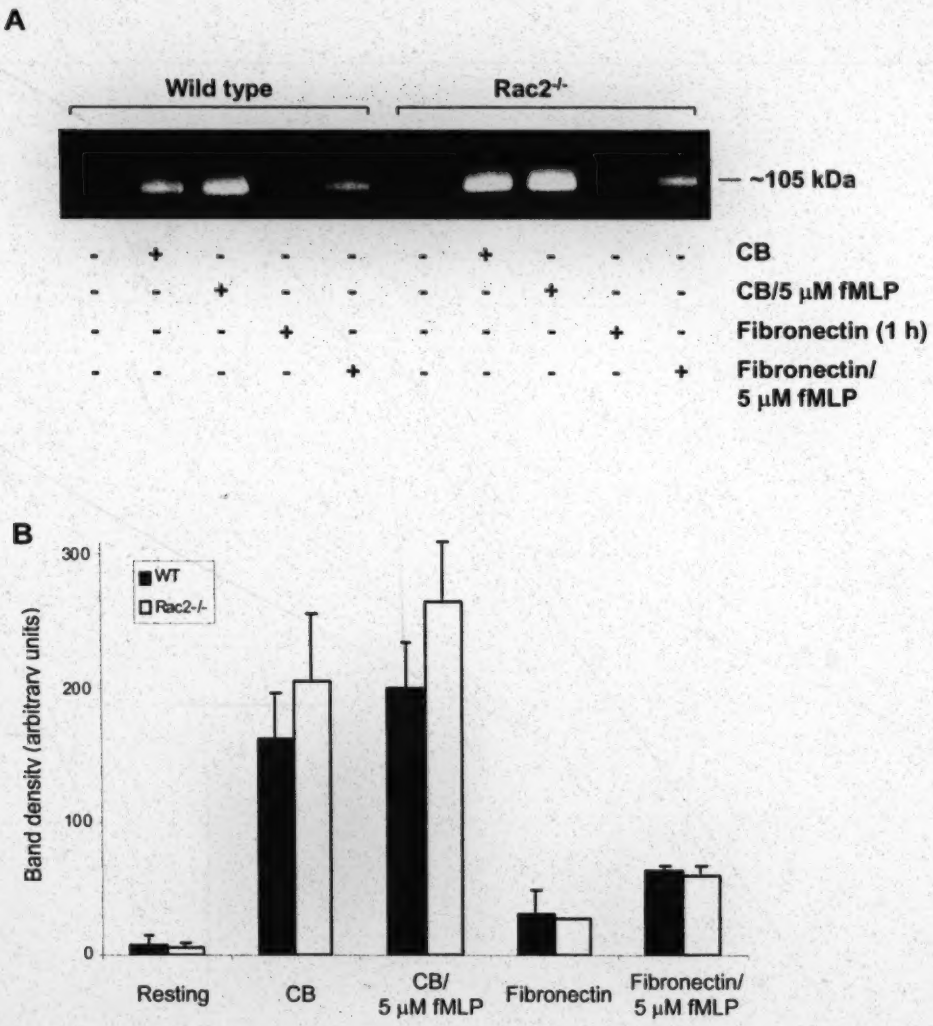


Figure 4.7. Release of tertiary granule MMP-9 is similar in WT and Rac2$^{-/-}$ neutrophils.

(A) Supernatants of WT and Rac2$^{-/-}$ BMN, either resting or stimulated with CB, CB/5 μM fMLP, fibronectin (1h), or fibronectin (1 h)/5μM fMLP, were separated by gel electrophoresis and analyzed by zymography to detect gelatinase activity, most of which migrated at ~105 kDa. (B) Graph showing the average optical density of MMP-9 activity in supernatants of cells stimulated by CB, CB/5 μM fMLP, fibronectin adhesion (1 h), or fibronectin (1 h) followed by 5 μM fMLP. (Figure 4.7 was generated by D. Abdel Latif)

Discussion

Rho GTPases are critical regulators of cellular activation events, which modulate the function of important intracellular effector molecules.[5] In this report, we show that Rac2 is required for primary granule release in neutrophils in response to chemoattractant stimulation. $Rac2^{-/-}$ neutrophils exhibited a profound defect in their ability to release two different primary granule mediators, MPO and elastase, in response to CB/fMLP and CB/LTB_4. In support of these findings, $CD63^{+}$ granules in $Rac2^{-/-}$ neutrophils failed to translocate in response to CB/fMLP, while WT cells exhibited intense granule mobilization to submembranous regions. Addition of the potent priming cytokine TNF did not rescue the degranulation defect in $Rac2^{-/-}$ neutrophils in response to CB/fMLP, although studies have shown that TNF augmented PMA-induced superoxide release and fMLP-induced F-actin formation in these cells.[19] Peritoneal exudate neutrophils from *rac2*$^{-/-}$ animals also showed no MPO release upon stimulation with CB/fMLP. Therefore, the defect in degranulation is not related to deficiencies in neutrophil priming. This is also the first report, to our knowledge, that describes primary granule mediator release in murine neutrophils.

The loss of primary granule release in $Rac2^{-/-}$ BMN is unrelated to granulogenesis defects, since their side scatter properties resembled that of WT BMN, and thin sections of $Rac2^{-/-}$ BMN examined by EM exhibited identical granularity to that of WT cells. Therefore, Rac2 appears to be specifically required for the intracellular machinery dedicated to neutrophil primary granule translocation prior to docking and fusion. Granule translocation is likely to be dependent on highly specific cytoskeletal remodelling events. Moreover, the release of secondary and tertiary granules was not

defective in CB/fMLP-treated Rac2$^{-/-}$ neutrophils, suggesting that cytoskeletal reorganization associated with exocytosis in these granule populations was intact in these cells. The addition of CB (an inhibitor of actin filament formation that acts as a priming reagent in neutrophils) is a prerequisite for chemoattractant-induced primary granule release. However, CB alone was able to induce secondary and tertiary granule release, indicating that degranulation can occur by CB-induced microfilament depolymerization.[39] Since CB is a nonphysiological agent, we carried out measurements of LTF and MMP-9 release in response to adhesion to fibronectin and fMLP, and found that the release of secondary and tertiary granules was similar in WT and Rac2$^{-/-}$ BMN. Our findings suggest that secondary and tertiary granule exocytosis is unimpeded in Rac2$^{-/-}$ BMN during physiological stimulation. Although it was not possible to compare the release of mediators from all three granule types in response to the same stimulus, the data shows that primary granule release in Rac2$^{-/-}$ neutrophils was abolished even during artificial or physiological priming.

These findings also indicate a lack of functional overlap between Rac1 and Rac2 in neutrophil primary granule exocytosis. Another study has recently suggested divergent roles for Rac1 and Rac2 in cell spreading and motility, respectively.[40] This is in contrast to previous studies which suggested overlapping roles for Rho GTPases in cytoskeletal reorganization, cell motility, and gene transcription.[5] Murine BMN from *rac2*$^{-/-}$ animals express Rho, Rac1, and Cdc42,[19] as determined by Western blot analysis (data not shown), and generate similar amounts of Rac1 and Rac2,[9] in contrast to human neutrophils which express predominantly Rac2.[8] No other Rho GTPase substituted for Rac2 in mediating primary granule release in spite of being expressed at levels similar to

WT neutrophils. Thus, downstream signals of Rac2 in this pathway are unlikely to cross-react with other GTPases. Based on our experimental data in this paper, we were unable to discriminate whether the defect in primary granule release in $Rac2^{-/-}$ neutrophils was in the signaling pathway adjacent to the receptor or in the final steps leading up to exocytosis, which will be important to determine in future investigations.

Rac2 has been shown to activate MAP kinases in other cell types.[41] In particular, p38 and ERK1/2 kinases have been proposed to act as effectors downstream of Rac2 signaling during fMLP stimulation. Moreover, studies have demonstrated that p38 MAP kinase is an obligatory effector molecule required for fMLP-induced IL-8 release in human neutrophils,[37] and that inhibition of p38 MAP kinase by SB203980 led to decreased primary and secondary granule release in CB/fMLP-stimulated human neutrophils.[14] Similarly, p38 MAP kinase activity was slightly decreased in fMLP-stimulated $Rac2^{-/-}$ neutrophils, suggesting that p38 MAP kinase may function downstream of Rac2.[19] In this study, we found that CB/fMLP induced strong p38 phosphorylation in $Rac2^{-/-}$ BMN, while TNF did not augment the phosphorylation signal induced by CB/fMLP. These findings suggest that activation of p38 MAP kinase is not related to the degranulation defect in $Rac2^{-/-}$ neutrophils, and that p38 activation may not be sufficient for primary granule release.

In contrast to the effects of *rac2*$^{-/-}$ gene deletion on primary granule release, secondary and tertiary granule release was intact in $Rac2^{-/-}$ BMN in response to CB/fMLP and adhesion to fibronectin. In addition, PMA induced LTF release from both WT and $Rac2^{-/-}$ BMN. The diacylglycerol-sensitive C2 domain serves as a target for phorbol ester stimulation, and proteins containing this domain include conventional isoforms of PKC.[42]

PMA induces superoxide production from neutrophils by activation of the NADPH oxidase complex through PKC stimulation.[43,44] However, PMA did not evoke primary granule release from murine bone marrow neutrophils. The discrepancy in the effects of PMA on superoxide production and primary granule exocytosis, and the observation that Rac2 is important in both processes, suggests that diacylglycerol-sensitive proteins, such as conventional PKC isoforms, may represent a split in signaling pathways downstream of Rac2 which have not yet been identified for primary granule exocytosis.

In summary, these findings indicate that Rac2 is a critical regulatory GTPase in primary granule exocytosis. The morphology of neutrophils from *rac2*$^{-/-}$ mice was similar to that of WT animals, suggesting that granulogenesis was normal in these cells. Priming of neutrophils and expression of other homologous Rho GTPases in these cells failed to substitute for the degranulation defect. These findings provide important insights into pathways regulating neutrophil exocytosis, which may serve as targets for anti-inflammatory therapy.

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Chapter 5

Neutrophil primary granule release and maximal superoxide generation depend on Rac2 in a common signaling pathway

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Figure 5.1 and 5.6 were contributed by Ms. Melissa Steward

Abstract

Neutrophils play an integral role in innate immunity by undergoing degranulation and respiratory burst in response to inflammatory stimuli. Rac2, a monomeric GTP-binding protein, has been shown to be involved in several neutrophil functions, including primary granule release and superoxide ($O_2^{\cdot-}$) generation. We hypothesize that Rac2 is a common signaling molecule required for granule translocation as well as maximal $O_2^{\cdot-}$ production. Using bone marrow neutrophils from Rac2 KO mice and wild type C57Bl/6 mice, we found that primary granule elastase and MPO release were absent in Rac2 KO neutrophils in response to chemoattractant stimulation. Rac2 KO neutrophils also failed to produce maximal levels of extracellular $O_2^{\cdot-}$ generation in response to phorbol myristate acetate (PMA). Although PMA was ineffective at eliciting primary granule mediator release, it induced secondary granule exocytosis in both WT and Rac2 KO neutrophils. Thus, the signaling pathway leading to primary granule release utilizes Rac2, which is also required for full activation of $O_2^{\cdot-}$ generation in stimulated neutrophils. These findings indicate that $O_2^{\cdot-}$ release and secondary granule secretion may utilize protein kinase C (PKC)-dependent pathways, while primary granule exocytosis appears to rely on PKC-independent signaling events. These findings shed light on possible signaling mechanisms involved in granule secretion from activated neutrophils in response to different stimuli.

Introduction

Neutrophils are terminally differentiated white blood cells produced in the bone marrow by hematopoiesis. Following recruitment to sites of inflammation, neutrophils undergo phagocytosis, mediator release, and respiratory burst. Over-activation of neutrophils, leading to mediator release, has been postulated to cause tissue damage in numerous diseases, such as acute respiratory distress syndrome, rheumatoid arthritis, and ischaemia-reperfusion injury. Neutrophil-derived mediators include granule products, secreted by degranulation, and reactive oxygen species (ROS).

At least four different types of granules can be observed in the cytoplasm of a mature neutrophil. These are primary, secondary, and tertiary granules and secretory vesicles, which contain numerous microbicidal mediators. Several mediators have been shown to localize specifically to neutrophil primary granules, including myeloperoxidase, elastase, and defensins (Borregaard and Cowland 1997). During degranulation, preformed granule mediators are released into extracellular and/or intracellular compartments. Elevations in intracellular Ca^{2+} has been shown to mediate a hierarchical release of neutrophil granules, where secretory vesicles are the first to fuse with the membrane, followed by tertiary and secondary granules, and finally primary granules (Borregaard and Cowland 1997). Patch clamp analysis and permeabilization studies have demonstrated that granule fusion requires, as a minimum, guanosine triphosphate (GTP) and Ca^{2+} (Nüsse and Lindau 1988).

Members of the Rho GTPase subfamily of *ras*-related GTPases, including Rac1, Rac2, and Cdc42, have been suggested as possible exocytotic GTPases (Brown et al. 1998). Rho GTPases are major regulators of diverse cell functions such as growth,

motility and development (Etienne-Manneville and Hall 2002). Containing at least 18 members, Rho-related GTPases are divided according to their homology to three prototypic proteins: Rac, RhoA, and Cdc42.

Superoxide anion ($O_2^{\cdot-}$) production is an important defence mechanism performed by neutrophils as part of their role in maintaining the innate immune response. The generation of $O_2^{\cdot-}$ requires membrane assembly of at least five subunits for the NADPH oxidase complex. This process involves association between cytochrome b_{558} subunits ($p22^{phox}$ and $gp91^{phox}$), residing in the granule/plasma membrane, with three cytosolic subunits: Rac, $p47^{phox}$, and $p67^{phox}$ (DeLeo and Quinn 1996). Rac1 and Rac2 are two members of the Rac subfamily that are functionally interchangeable in their ability to activate NADPH oxidase. Rac2 exhibits 92% homology to Rac1 in its amino acid sequence (Knaus et al. 1991). However, they have distinct tissue distribution patterns; thus, while Rac1 is ubiquitously expressed, Rac2 expression is restricted to cells of the haematopoietic lineage (Didsbury et al. 1989).

Bone marrow neutrophils isolated from Rac2 knockout (KO) mice exhibit a profound loss of chemotaxis in response to different stimuli (Roberts et al. 1999). Stimulation of these cells with f-Met-Leu-Phe (fMLP), tumor necrosis factor, and phorbol myristate acetate (PMA) failed to induce maximal $O_2^{\cdot-}$ production (Roberts et al. 1999). Furthermore, $Rac2^{-/-}$ neutrophils exhibited a deficiency in primary granule release in response to chemoattractant stimuli (Abdel-Latif et al. 2004).

We hypothesize that neutrophil primary granule release and maximal $O_2^{\cdot-}$ production depend on Rac2 in a common signaling pathway. Bone marrow neutrophils (BMN) were harvested from Rac2 KO and wild type (WT) mice to investigate

degranulation and $O_2^{\cdot-}$ production in response to different stimuli. We show that ROS production did not alter the activity of primary granule MPO released from activated neutrophils. We also report that PMA was ineffective at inducing maximal $O_2^{\cdot-}$ generation and primary granule mediator release, while it induced normal levels of secondary granule mediator release and partial $O_2^{\cdot-}$ production.

Methods

Materials. Chemicals were purchased from Sigma-Aldrich Co. (Oakville, ON) unless otherwise noted. Ca^{2+}, Mg^{2+}, phenol red-free Hank's balanced salt solution (HBSS), and phosphate-buffered saline (PBS, pH 7.2) were obtained from Life Technologies (Grand Island, NY). HBSS (pH 7.4) was supplemented with BSA (0.1%) and glucose (1%). Phenol red-free RPMI-1640 was purchased from Life Technologies.

Animals. The *rac2* KO mice used in this study were previously generated by targeted disruption of the *rac2* gene (Roberts et al. 1999), which had been backcrossed into C57Bl/6 mice for more than 11 generations. Wild-type C57Bl/6 control mice were from Charles River Canada, Saint-Constant, PQ. Animals were housed under specific pathogen-free conditions and fed autoclaved food and water as needed. Mice used in these experiments were between 8 – 20 weeks of age.

Preparation of bone marrow neutrophils. BMN were obtained by density gradient centrifugation using a modification of previously described techniques (Lowell et al. 1996; Kim and Dinauer 2001). Briefly, BMN were isolated from femurs and tibias flushed with 3 ml of HBSS-BG using a 22-gauge needle (Becton Dickinson, San Jose, CA). Cells were pelleted by centrifugation at 300*g* for 10 min at 4°C before mixing with 45% Percoll (Pharmacia, Uppsala, Sweden) and layering onto gradients consisting of 3 ml 81%, 2 ml 62%, 2 ml 55%, and 2 ml 50% Percoll in HBSS-BG. After centrifugation of gradients at 600*g* for 30 min at 6°C, BMN were removed from the interface between the 62% and 81% layers and washed twice with HBSS-BG. To remove contaminating red blood cells, BMN were centrifuged on Histopaque 1119 at 600*g* for 30 min at 6°C, and washed twice in HBSS-BG. The purity of neutrophils obtained in these preparations

averaged 60-70%, as determined by Diff-Quik staining, which is similar to previously published values (Kim and Dinauer 2001). Neutrophil viability was >90% as determined by trypan blue exclusion.

RT-PCR of murine BMN. Cells were subjected to total RNA extraction using a Qiagen RNeasy MiniKit (Qiagen, Mississauga, Canada) using 1 x 10^6 cells per extraction. Primer sequences used for detecting Rac1or Rac2 mRNA were generated in our laboratory for Rac1 (product size, 575 bp), with forward and reverse sequences as follows: (2–24 bp) , 5'-TGCAGGCCATCAAGTGTGTGGTG-3' and (554–576 bp) 5'-CAACAGCAGGCATTTTCTCTTCC-3'. Rac2-specific primers (product size, 576 bp) were (3–25 bp) 5'-GCAGGCCATCAAGTGTGTGGTGG-3' and (556–578 bp) 5'-TAGAGGAGGCTGCAGGCGCGCTT-3', respectively. Both sets of primers were intron spanning (Courjal et al. 1997; Matos et al. 2000). Reactions were carried out in a PTC 100 Thermal Controller (M-J Research, Watertown, MA) using an annealing temperature of 57°C for Rac1-specific primers and 56°C for Rac2-specific primers. Non-reverse-transcribed samples were included as controls.

Western Blotting. Samples were subjected to acrylamide gel electrophoresis and transferred to Immobilon polyvinylidene difluoride membrane blots followed by incubation of the membrane with monoclonal anti-Rac antibody (Upstate Biotechnology), polyclonal rabbit anti-human Rac2 (courtesy of G. Bokoch), and monoclonal anti-Cdc42 (BD Transduction Laboratories).

Degranulation assays. BMN were washed and resuspended at 5-10 x 10^6 cells/ml in phenol red-free RPMI-1640 for all degranulation assays, which were carried out on cells in suspension. Aliquots (50 µl) of cells were preincubated with 5 µg/ml cytochalasin B

(CB) or dihydrocytochalasin B (DHCB) for 5 min at 37°C before adding increasing doses of fMLP (0-40 μM) or leukotriene B_4 (LTB_4, 0-1 μM). Cells were incubated for a further 15 min (fMLP) or 1hr (LTB_4) before subjecting them to centrifugation at 4°C for 5 min at 300*g*. PMA stimulated neutrophils were preincubated with (0-500 ng/ml) for 15 minutes at 37°C. Supernatants were removed to assay for released granule proteins, while resting cells were lysed by the addition of an equivalent volume of RPMI-1640 containing 0.5% CHAPS. For superoxide dismutase (SOD) preincubation, 50 U/ml of SOD were incubated with BMN at 37°C for 5 min prior to the addition of CB and fMLP.

Neutrophil elastase (NE) activity. NE activity was measured using an EnzCheck elastase assay kit (Molecular Probes, Eugene, OR). Briefly, 100 μl of fresh sample was incubated with 100 μl substrate solution (DQ elastin labelled with BODIPY FL dye) for 24 h at room temperature in the dark (shorter incubations generated less fluorescence) before reading the resulting fluorescence at 505 nm for excitation and 515 nm for emission. Release of elastase was calculated as a percentage of total cellular activity by dividing the corrected absorbance of supernatants into the sum of supernatants and lysed pellets.

Myeloperoxidase secretion. MPO was assayed using tetramethylbenzidine based on a previously established technique (Lacy et al. 1999). Briefly, 150 μl of tetramethylbenzidine substrate solution was added to 50 μl of sample and incubated at ambient temperature for 30 minutes prior to termination with 50 μl of 1 M H_2SO_4. Plates were read spectrophotometrically at 450 nm (Molecular Devices, Sunnyvale, CA). Absorbance values for released MPO and elastase were divided into the average of values from 0.5% CHAPS-lysed cells to give the percentage of total cellular MPO content.

Measurement of $O_2^{\cdot-}$ release from neutrophils. Generation of extracellular $O_2^{\cdot-}$ from cells in suspension was measured as previously described (Lacy et al. 2003). SOD-inhibitable absorbance was calculated using 2.11×10^4 M^{-1} cm^{-1} for reduced cytochrome *c*.

Statistical analysis and data presentation. Data were analyzed by two-tailed Student's *t*-test or one-way ANOVA analysis followed by Tukey's column comparison, and depicted in figures as mean ± SEM.

Results

Absence of Rac2 expression in Rac2 KO neutrophils. To confirm Rac2 gene deletion in BMN obtained from Rac2 KO mice, primers detecting Rac1 and Rac2 were employed (Lacy et al. 2003). Rac1 expression in WT and Rac2 KO BMN was investigated by RT-PCR analysis using human neutrophils as the positive control (Figure 5.1). Rac2 KO BMN expressed Rac1 mRNA, similarly to WT BMN. However, Rac2 mRNA expression was absent in Rac2 KO BMN, while WT BMN showed a PCR product of equivalent size to that of human neutrophils (Figure 5.1).

An assessment of Rac1 and Rac2 protein expression was carried out by Western blot analysis of whole cell lysates from WT and Rac2 KO BMN (Figure 5.2). Rac1 overproduction was evident at the level of protein expression in Rac2 KO BMN, which has been shown previously and may represent a compensatory mechanism for the loss of Rac2 expression (Li et al. 2002). WT BMN showed Rac2 protein expression, while Rac2 KO neutrophils failed to react with anti-Rac2 antibody, indicating little expression of Rac2 protein. We also investigated the expression of Cdc42, another Rho GTPase that may play a role in primary granule release from activated neutrophils. Blots incubated with anti-Cdc42 showed equivalent levels of expression of Cdc42 in both Rac2 KO and WT bone marrow neutrophils (Figure 5.2).

Primary granule release is abolished in Rac2 KO neutrophils. A fluorometric assay was carried out to determine the effect of LTB_4 on elastase release from Rac2 KO BMN. Neutrophils were stimulated with CB/LTB_4 in increasing doses, and supernatants were assayed for elastase activity. WT BMN displayed normal NE secretion in response to CB/LTB_4 (Figure 5.3). However, Rac2 KO BMN supernatants were devoid of elastase

activity (Figure 5.3). This finding supports our previous observation showing an essential role for Rac2 in primary granule release in response to chemoattractant stimuli (Abdel-Latif et al. 2004).

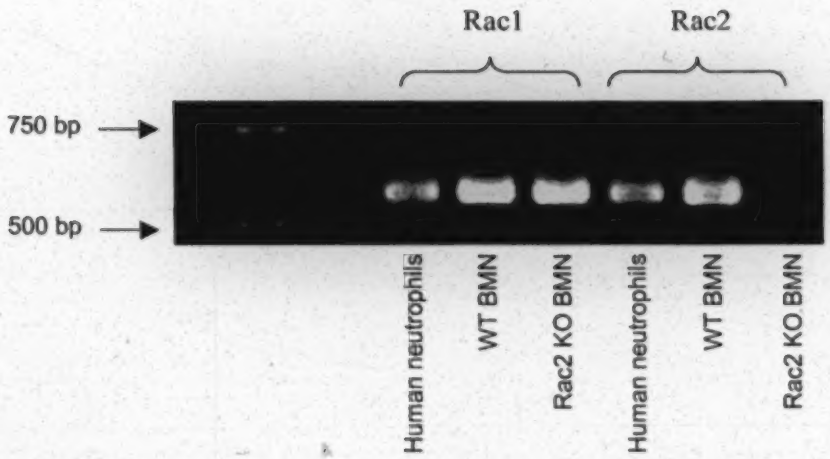

Figure 5.1. Absence of Rac2 mRNA expression in Rac2 KO BMN.
BMN prepared from WT and Rac2 KO mice were examined for Rac2 mRNA expression using RT-PCR. The Rac2 PCR product was absent from Rac2 KO BMN, but present in WT cells at a similar size to human neutrophils. Rac1 PCR products were of equivalent sizes in WT BMN, Rac2 KO BMN, and human neutrophils. (Figure 5.1 was generated by M. Steward)

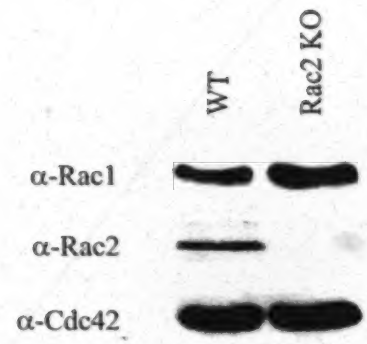

Figure 5.2. Rac2 KO neutrophils express Rac1 and Cdc42.
Whole cell lysates of WT and Rac2 KO BMN were prepared and loaded on 16% polyacrylamide gels to resolve bands of similar molecular weights. Blots were probed with (A) a monoclonal anti-Rac antibody (cross-reactive for both Rac1 and Rac2), (B) a polyclonal anti-Rac2 antibody (which specifically recognizes Rac2 and not Rac1), and (C) a monoclonal anti-Cdc42 antibody. (Figure 5.2 was generated by D. Abdel Latif)

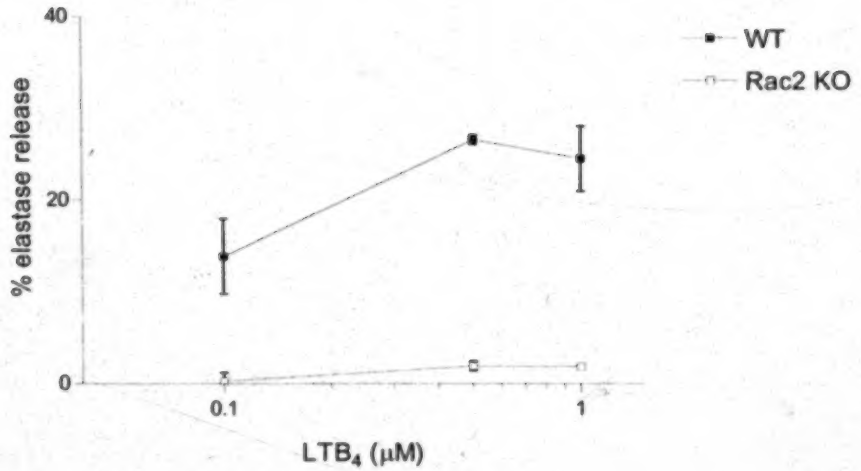

Figure 5.3. NE release is abolished in Rac2 KO BMN.
Freshly prepared BMN from WT and Rac2 KO were assayed for NE (a primary granule marker) release in response to increasing doses of LTB_4 for 1 hour following 5 min preincubation with 5 μg/ml CB. (Figure 5.3 was generated by D. Abdel Latif)

CB effects on monosaccharide transport do not alter primary granule release. We have shown that CB/LTB_4 induces elastase release in WT but not Rac2 KO neutrophils. However, CB may inhibit monosaccharide transport in the cell membrane and adversely influence degranulation events. Thus, DHCB (5 μg/ml) was substituted for CB as a more specific inhibitor of actin polymerization. Incubation of WT BMN with DHCB/fMLP (0.1-40 μM) resulted in normal release of MPO, while Rac2 KO BMN did not release MPO under these conditions (Figure 5.4). These data suggest that the deficiency in primary granule mediator release from CB/LTB_4-treated Rac2 KO neutrophils was not due to inhibition of monosaccharide transport by CB in these cells.

ROS production by activated neutrophils does not affect MPO activity. The production of ROS by neutrophils during CB/fMLP stimulation may catalyze MPO oxidation and alter its activity (Clark and Borregaard 1985). Cells were preincubated with SOD to counteract the potential effects of $O_2^{\cdot-}$ on released MPO activity. However, SOD preincubation did not alter detectable MPO release in supernatants in either WT or Rac2 KO BMN, suggesting that the effects of ROS were minimal on secreted MPO activity (Figure 5.5).

Rac1 is unable to substitute for Rac2 in eliciting BMN maximal $O_2^{\cdot-}$ production. In order to establish the responsiveness of Rac2 KO neutrophils to respiratory burst stimulation, BMN from *rac2* KO and WT mice were tested using the conventional cytochrome *c* reduction assay. BMN from *rac2* KO mice generated 53% less $O_2^{\cdot-}$ than WT controls in response to stimulation with 500 ng/ml PMA (Figure 5.6), correlating with previously published data (Roberts et al. 1999). Although murine neutrophils preferentially utilize Rac2 for activation of the NADPH oxidase complex, these cells also express Rac1, which partially compensates for deficiencies in Rac2 functions (Li et al. 2002). This finding

suggests that maximal $O_2^{\cdot-}$ generation depends on Rac2, and that Rac1 alone cannot achieve optimal $O_2^{\cdot-}$ production during PMA stimulation of neutrophils.

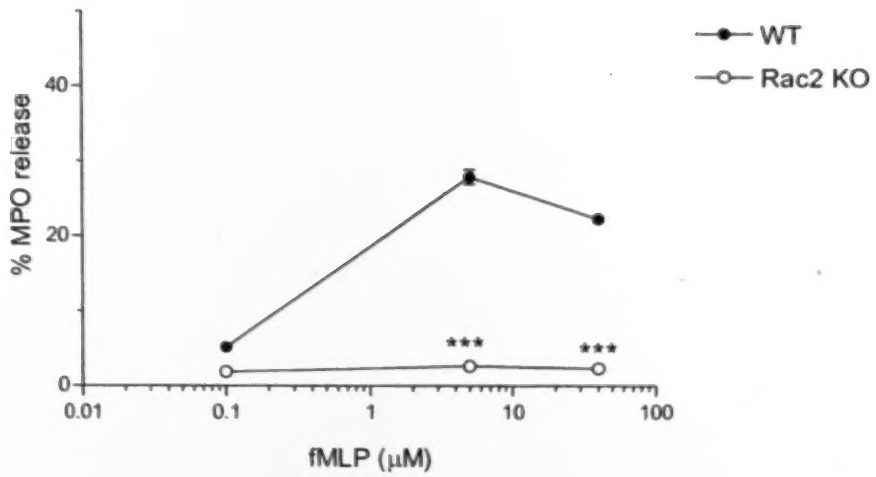

Figure 5.4. Rac2-deficient BMNs fail to secrete MPO in response to DHCB/fMLP. Freshly prepared BMN from WT and Rac2 KO neutrophils were examined for their ability to release the primary granule marker MPO in response to increasing doses of fMLP for 15 minutes following 5 minutes preincubation with 5 μg/ml DHCB. MPO release was determined as a percentage of total cell MPO content. * indicates $p < 0.05$, ** $p < 0.02$, and *** $p < 0.01$. (Figure 5.4 was generated by D. Abdel Latif)

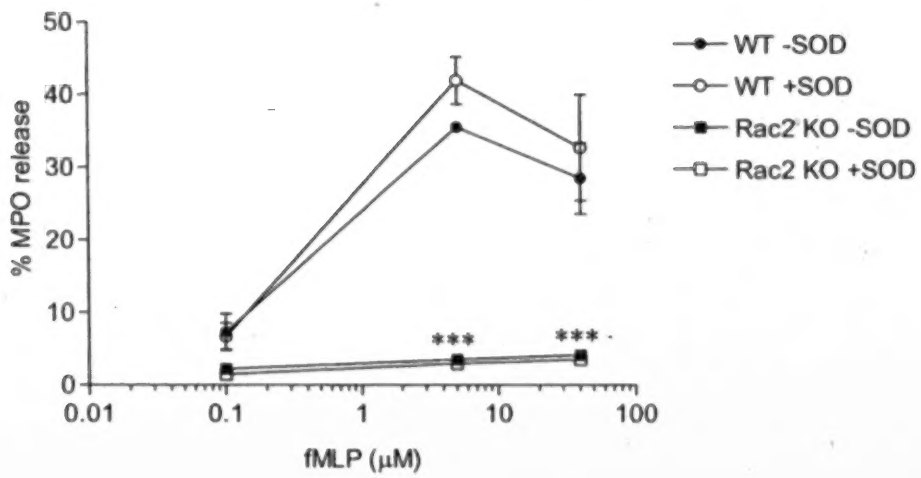

Figure 5.5. Preincubation with SOD did not alter MPO activity in supernatants. WT and Rac2 KO BMN were stimulated with CB/fMLP with preincubation of 50 U/ml SOD for 5 min. *** indicates $p < 0.01$. (Figure 5.5 was generated by D. Abdel Latif)

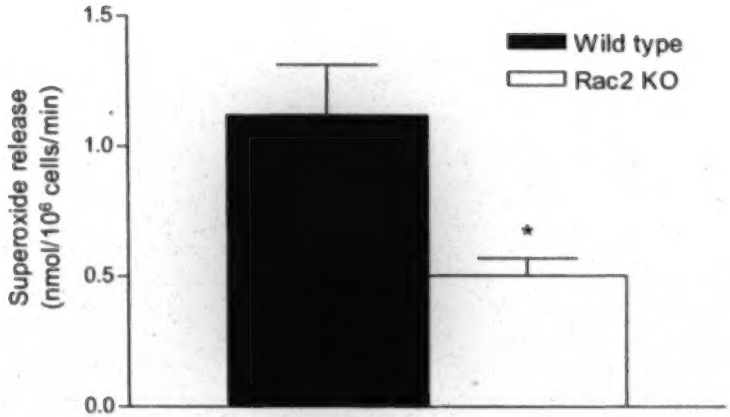

Figure 5.6. Maximal $O_2^{\cdot-}$ generation is mediated by Rac2.
Freshly isolated BMN were stimulated with PMA (500 ng/ml) and $O_2^{\cdot-}$ production was measured using a cytochrome *c* reduction assay. $O_2^{\cdot-}$ generation was inhibited in Rac2 KO neutrophils in response to PMA, in comparison to WT neutrophils. * indicates $p < 0.05$. (Figure 5.6 was generated by M. Steward)

PMA stimulation induces secondary but not primary granule mediator release. The effect of phorbol esters on primary and secondary granule release was also assessed. We found that MPO secretion in response to PMA was negligible in both WT and Rac2 KO BMN (Figure 5.7A), indicating that primary granule release is unlikely to be dependent on diacylglycerol-binding C2 domain-containing proteins such as conventional protein kinase C (PKC) isoforms. In contrast, PMA induced significant release of LTF from Rac2 KO neutrophils, which was similar to that released by WT BMN (Figure 5.7B). Thus, secondary granule exocytosis may utilize a signaling pathway similar to that used for $O_2^{\cdot-}$ production, involving PKC-dependent phosphorylation events.

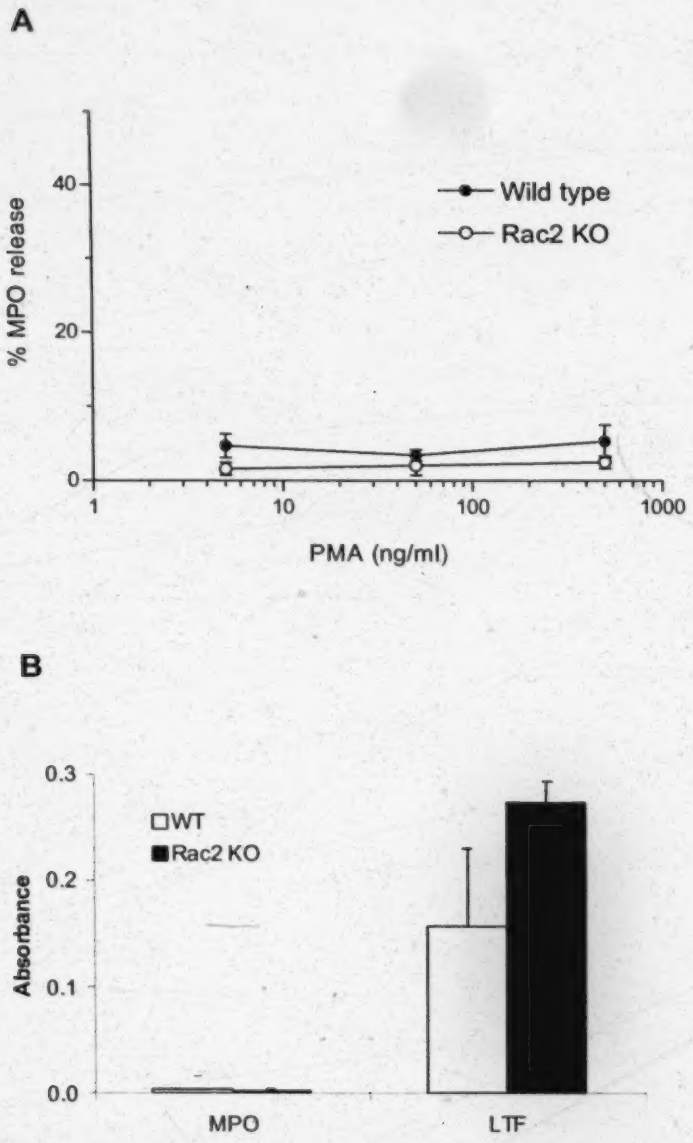

Figure 5.7. Secondary granule LTF release is equivalent in WT and Rac2 KO neutrophils in response to PMA.
BMN were stimulated with increasing doses of PMA and their LTF release determined by immunoassay. These figures show (A) MPO secretion in PMA-stimulated cells shown as release in percentages of total cellular MPO, and (B) LTF release from PMA-stimulated cells shown in absorbance values. (Figure 5.7 was generated by D. Abdel Latif)

Discussion

The Rho family of GTP-binding proteins are essential regulators of cellular functional events, and function by controlling the activation/inhibition status of important intracellular effector molecules (Ridley 2001). In this study, we demonstrated that neutrophil elastase release and maximal $O_2^{\cdot-}$ production depend on Rac2 in response to chemoattractant stimulation. Rac1 and Cdc42 expression were intact in Rac2 KO neutrophils, as shown by Western blot analysis. Hence, Rac1 and Cdc42 were unable to substitute for Rac2 function in primary granule release and optimal $O_2^{\cdot-}$ production in Rac2 KO neutrophils.

The discrepancy between Rac1 and Rac2 functions in neutrophil activation is very interesting, since these molecules exhibit 92% amino acid identity with each other and differ only in the final ~10 amino acids in the C-terminus of their protein sequences. The inability of Rac1 to substitute fully for Rac2 in $O_2^{\cdot-}$ production is likely to be due to the comparatively reduced affinity of Rac1 for the NADPH oxidase complex (Li et al. 2002). Our finding that primary granule release is abolished in Rac2 KO neutrophils reflects the distinct activation and signaling profiles of Rac1 and Rac2.

Neutrophil elastase is important for facilitating neutrophil migration and microbial killing. However, uncontrolled NE secretion may lead to tissue destruction and loss of vascular wall integrity. This study investigated the secretory profile of NE in Rac2 KO neutrophils as a result of CB/LTB_4 stimulation. A fundamental defect in NE secretion was observed in Rac2 KO BMN. These findings confirm our earlier study showing a loss of MPO and elastase release in response to CB/fMLP (Abdel-Latif et al. 2004). We have also ruled out the possibility that defects in granulogenesis may be responsible for the

failure in primary granule secretion. This was shown in thin sections of Rac2 KO BMN examined by electron microscopy, which exhibited identical granularity to that of WT cells, and in the similarity of forward and side scatter profiles by flow cytometry between WT and Rac2 KO BMN (Abdel-Latif et al. 2004). In addition, lysed cells exhibited similar quantities of stored MPO and elastase in WT and Rac2 KO BMN. Hence, *rac2* gene deletion did not interfere with granulogenesis, and instead appears to have altered the intracellular machinery dedicated to neutrophil primary granule release. The failure of degranulation in Rac2 KO neutrophils may be attributable to the lack of granule translocation to submembranous regions prior to docking and exocytosis. This defect was specific for primary granules rather than other granule populations (secondary and tertiary granules) in Rac2 KO neutrophils (Abdel-Latif et al. 2004). Taken together, our results indicate that primary granule exocytosis was abolished as a result of Rac2 deficiency, and that Rac2 plays a central role in primary granule exocytosis.

Primary granule release from WT neutrophils could not be obtained in the absence of cytochalasin-induced priming (Abdel-Latif et al. 2004). Cytochalasins are a group of fungal metabolites that permeate cell membranes and inhibit actin cytoskeleton polymerization to form F-actin from the soluble G-actin in the cytosol. Inhibition of F-actin formation appears to mimic cell adhesion events in neutrophils and enhance degranulation. However, cytochalasins A and B also inhibit monosaccharide transport across the plasma membrane. CB has been shown to have significant effects on glucose transport in erythrocytes (Bloch 1973; Taverna and Langdon 1973; Taylor and Gagneja 1975). In contrast, cytochalasins C, D, E, H and DHCB do not interfere with monosaccharide transport (Rampal et al. 1980). For this reason, we used DHCB to

substitute for CB. We were able to show that MPO release in response to DHCB/fMLP was similar to that released in response to CB/fMLP stimulation in our previous study (Abdel-Latif et al. 2004), suggesting that the effects of CB on degranulation (or the lack of it) did not arise from inhibition of glucose transport.

ROS generation by activated neutrophils may lead to oxidation of MPO in supernatants and subsequently diminish MPO activity (Clark and Borregaard 1985). Hence, we examined the effect of SOD on MPO detection levels from WT and Rac2 KO BMN. The addition of SOD prevents oxidative autolysis of proteins by neutralizing $O_2^{\cdot-}$ anions. Our results showed an insignificant effect of $O_2^{\cdot-}$ removal on MPO activity, suggesting that ROS production did not prevent the detection of MPO activity in supernatants from WT or Rac2 KO BMN.

Interestingly, PMA induced $O_2^{\cdot-}$ generation and secondary granule release, but was unable to evoke primary granule exocytosis from WT BMN. This suggests that intracellular targets of PMA differentially activate specific pathways of mediator release from neutrophils. Intracellular PMA targets are likely to include conventional PKC isoforms, as these have been shown to be involved in the induction of $O_2^{\cdot-}$ production from neutrophils (Lopes et al. 1999). The lack of effect of PMA on primary granule exocytosis suggests that PKC-mediated phosphorylation of intracellular effector molecules is not involved in this process.

In summary, Rac2 is a common molecule in neutrophils that is utilized by both degranulation and $O_2^{\cdot-}$ generation pathways. Two other Rho GTPases were shown to be unable to compensate for Rac2 in primary granule release. While partial compensation occurred in $O_2^{\cdot-}$ production, failure to reach a maximal response to PMA was observed.

Thus, Rac2 is a crucial molecule regulating a diversity of neutrophil functions and may be a candidate for pharmacological studies aimed at treatment of neutrophil-mediated inflammatory disorders.

Acknowledgments

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Chapter 6

Preliminary data

Rac2 deficiency protects against immune complex-mediated acute lung injury

Dalia Abdel Latif, Chris St Laurent, Lakshmi Puttagunta, Dean Befus and Paige Lacy

Figure 6.2 was generated with the assistance of Dr. Lakshmi Puttagunta.

Abstract

Neutrophils are key contributors to tissue destruction in acute lung injury (ALI). Tissue destruction in ALI is mediated by oxidative damage and degranulation. Rac2 is required for primary granule release, superoxide production and chemotaxis in neutrophils. We hypothesized that Rac2-mediated neutrophil activation induces tissue destruction in ALI. Following immune complex-mediated ALI, total cell and neutrophil counts in bronchoalveolar lavage fluid (BALF) from $Rac2^{-/-}$ mice were reduced compared with wild type (WT) animals. Myeloperoxidase, a primary granule marker, was undetectable in $Rac2^{-/-}$ BALF, while a significant increase was seen in WT BALF. Lactoferrin levels were equivalent in WT and $Rac2^{-/-}$ BALF, suggesting that its secretion from activated neutrophils and airway epithelium of both WT and *rac2*$^{-/-}$ mice during ALI. BALF protein and albumin concentrations were elevated in WT mice with ALI, but did not differ significantly from control animals and *rac2*$^{-/-}$ mice, indicating interference in tissue destruction cascades due to Rac2 deficiency. Histopathological analysis of the lungs from WT mice with ALI showed an abundant neutrophilic infiltration in the parenchyma, with accompanying hemorrhage and loss of lung architecture. In *rac2*$^{-/-}$ mice, neutrophilic infiltration was also evident but without areas of hemorrhage or loss of lung architecture. Therefore, lung tissue injury was substantially diminished in *rac2*$^{-/-}$ mice. We conclude that Rac2 plays a major role in inducing tissue damage in immune complex-mediated ALI.

Introduction

Acute lung injury (ALI) is a syndrome of acute respiratory failure accompanied by hypoxemia. Radiography of the lungs of ALI patients reveals a picture similar to pulmonary edema where bilateral radioopaque alveolar infiltrates show a patchy distribution. [1] ALI and its more severe form, adult respiratory distress syndrome, are two major causes of morbidity and mortality. An inflammatory aetiology for ALI has been heavily studied and a phagocyte-dependent tissue injury mechanism was proposed. [2-4]

Immune complex (IC)-mediated acute lung injury is a complex process that involves a number of different cell populations and their mediators. The inflammatory cascade starts with engagement of the immunoreceptor tyrosine-based activation motif (ITAM)-containing γ chain-associated Fc receptors on alveolar macrophages. [5-7] This in turn leads to the activation of alveolar macrophages and release of tumor necrosis factor (TNF), interleukin 8 (IL-8), macrophage inflammatory protein-1α (MIP-1α), MIP-1 β, regulated upon activation, normal T cells, expressed and secreted (RANTES), and monocyte chemoattractant protein 1 (MCP-1). [8,9] TNF induces the upregulation of selectins and intracellular adhesion molecule-1 (ICAM-1) on vascular endothelial cells. These factors facilitate the chemoattraction and extravasation of neutrophils at sites of IC deposition, reaching its peak at 4 h. [10] IC-mediated tissue injury mainly results from activated neutrophils [3,4] through the production and release of inflammatory cytokines (TNF, IL-1β), reactive oxygen species (ROS, including $O_2^{\cdot-}$ and its dismutated product, H_2O_2), and toxic mediators stored in the primary granules such as myeloperoxidase and elastase (MPO, NE). [2] Neutrophil chemotaxis, ROS production and the selective release of primary granules are regulated by Rac2, [11,12] a monomeric GTP-binding protein that is

exclusively expressed in hematopoietic tissues and belongs to the Rho family of GTPases. [13] Upon neutrophil stimulation, the inactive form of Rac2 (Rac2-GDP) is activated through guanine nucleotide exchange factor (GEF)-mediated binding to GTP. Rac2-GTP mediates neutrophil chemotaxis through promoting actin polymerization at the leading edge of the membrane, which is a pivotal step in cell movement. Furthermore, Rac2-GTP translocates to the cell or granule membrane and joins other components of the superoxide-generating NADPH oxidase system to activate the production of ROS. [14] Finally, Rac2 has been shown to control the release of toxic mediators from primary granules upon chemoattractant stimulation in neutrophils [12] and from secretory granules following IgE crosslinking in mast cells. [15] Thus, a deficiency of Rac2 in neutrophils is expected to reduce inflammation in diseases that involve acute or chronic inflammatory reactions.

In order to characterize the effect of Rac2 deficiency on neutrophil-mediated lung inflammation, we utilized an IC- mediated model of acute lung injury. Upon deposition of immune complexes in the lung, a severe inflammatory reaction occurred in wild type mice with increased vascular permeability, loss of pulmonary architecture, and hemorrhage. However, in $Rac2^{-/-}$ mice, these features were absent. In this study we observed that the loss of Rac2 expression leads to the attenuation of tissue destruction and prevention of lung injury in this model.

Materials & Methods

Mice. The generation and phenotype of *rac2*$^{-/-}$ mice is previously reported. [11] All gene-deleted mice were backcrossed against the C57Bl/6 strain background for more than 11 generations. Wild-type C57Bl/6 mice were obtained from Charles River, Saint-Constant, PQ, Canada. Animals were housed under specific virus antigen-free conditions and fed autoclaved food and water as needed. Mice (males and females) used in these experiments were between 8 – 40 weeks of age. The University of Alberta Health Sciences Animal Policy and Welfare Committee approved all animal related techniques in accordance with guidelines of the Canadian Council for Animal Care.

Immune complex-mediated acute lung injury. Isoflurane inhalation using a precision vaporizer instrument (Ohio Medical Corporation, Gurnee, IL, USA), and intraperitoneal (i.p.) injections of ketamine and xylazine (1.5 mg and 150 μg/ mouse, respectively) were used for anesthesia and analgesia respectively. The reverse passive Arthus reaction was induced in the airways of the animal by intratracheal (i.t.) injection of 16 μg/mouse of rabbit anti-ovalbumin IgG (Valeant Pharmaceuticals International, Costa Mesa, CA, USA) in water, followed by a tail injection of 400 μg of ovalbumin (Sigma Chemical Co., Oakville, ON, Canada) dissolved in saline. Control mice were C57Bl/6 wild type and *rac2*$^{-/-}$ animals that did not receive any treatment, while antibody controls received an i.t. injection of 16 μg rabbit anti-ovalbumin IgG. Mice were sacrificed six hours after i.t. and tail vein injections by an i.p. overdose of ketamine and xylazine (15 mg and 1.5 mg/ mouse, respectively). We employed an incubation period of 6 h from the time of antigen injection to account for the lag period consumed by antigen delivery to the lungs with resulting IC deposition. Furthermore, it was shown previously

that injury is initiated in this model at 4 h, reaches its maximum at 8 h, and plateaus until 24 h. [16] C57BL/6 wild type mice were used as a positive control for IC deposition in the lungs upon OVA/anti-OVA injection. Dose and time point optimization took place in the wild type mice followed by implication of the knowledge acquired in the Rac2 deficient mice. To determine if $Rac2^{-/-}$ mice have a delay in IC deposition in the lung, a 24 h treatment was performed that showed similar data to those observed at 6 h treatment (data not shown).

Lung histology. For murine lung histology, adult C57Bl/6 mice with or without immune complex-induced lung injury were sacrificed, and lungs were exposed by opening the chest cavity by a parasagittal incision. Tracheae were cannulated and 1-1.5 mls of 10% buffered formalin was used to inflate the lungs, followed by fixation in the same solution for 24 hours. The entire lungs were submitted for tissue processing, where they were embedded in paraffin and sectioned at 4 μm followed by hematoxylin and eosin staining. Images were acquired using a Nikon Eclipse model E600 configured for standard bright field microscopy and an 11-mega pixel Nikon DXM 1200 digital camera attached to the microscope and employing control software, Nikon Act-1, (Nickon Corporation. Mississauga, ON, Canada) installed on a PC running Windows XP.

Broncho-alveolar lavage fluid (BALF) collection. Tracheae were exposed by trans-salivary incision and cannulated with a 24-gauge needle, and lavage was performed with 1 ml ice-cold PBS, which was then collected in maximum recovery tubes (Axygen Scientific, Inc., Union City, CA, USA) and placed on ice. This process was repeated 6 times. All fractions of collected PBS were spun down at 300 *g* and 4°C for 5 min to pellet inflammatory cells. Pelleted cells were counted by hemocytometer and Kimura's stain to

determine total cell counts. Cytospins were prepared and stained with Diff-Quik stain to measure the differential cell counts in BALF. Supernatants were divided into two portions; 300 µl was stored at -80°C for future lactoferrin (LTF), matrix metalloprotease-9 (MMP-9), mouse albumin, and cytokine analysis; while the remainder was immediately analyzed for MPO.

Myeloperoxidase assay. MPO was assayed using tetramethylbenzidine (TMB) as the substrate, as previously described. [17] Briefly, 150 µl of TMB substrate solution was added to 50 µl of BALF in a 96-well microplate and incubated at room temperature for 15 minutes prior to termination of the reaction with 50 µl of 1 M H_2SO_4. Microplates were read spectrophotometrically at 450 nm using a Power Wave XS plate reader (Bio-Tek Instruments, Inc, Winooski, VT, USA). Absorbance values for background (PBS only) were subtracted from sample MPO values.

Lactoferrin (LTF) ELISA. LTF was assayed in BALF by ELISA measurement based on cross-reactivity of human anti-LTF (Sigma-Aldrich, Oakville, ON, Canada) for murine LTF. [18,18] Briefly, supernatants were diluted 1:10 in bicarbonate buffer (50 mM $NaHCO_3$, 16.8 mM Na_2CO_3 in H_2O, pH 9.6) according to previously published protocol [12] and incubated on Nunc Maxisorp plates (VWR International, Ltd., Mississauga, ON, Canada) for 1 h, followed by washing and incubation with anti-human LTF for 1 h. Blocking with 1% BSA preceded the addition of secondary antibody, horseradish peroxidase (HRP)-conjugated anti-rabbit antibody(Amersham Biosciences, Piscataway, NJ, USA), which was left to incubate for 1 h. Plates were washed and incubated with TMB substrate solution for 15 min. Plates were read using a Power Wave XS plate reader (Bio-Tek Instruments, Inc). All incubations for this assay were done at room temperature.

Gelatin zymography for matrix metalloproteinase activity. BALF samples (10 μL) were subjected to gel electrophoresis in 1% gelatin-containing polyacrylamide gels and analyzed for gelatinase activity as previously described. [12,19] Samples were added to 4X zymography loading buffer (40% glycerol, 8% SDS, 20 μg/ml bromophenol blue in 0.25 M Tris, pH 6.8) loaded on 10% acrylamide ready-made zymogram gels (BioRad Laboratories, Hercules, CA). Electrophoresis was carried out at 160 mV for 2 h and gels were washed 3 times for 20 min with 2.5% Triton X-100 at room temperature with shaking. Gels were incubated with zymography buffer (0.9% NaCl, 5 μM $CaCl_2$, 0.00025% NaN_3 in 50 mM Tris) for up to 1 week at 37°C. Gel staining was carried out using 0.5% Coomassie blue G-250 in 25% methanol, 10% acetic acid for 1 h with shaking at room temperature, followed by destaining overnight with 4% methanol and 8% acetic acid. . Mouse MMP-9 is expected to appear at~ 105 kDa, while we used PMA stimulated HT 1080 (human fibrosarcoma cell line) as a positive control as previously described. MMP-9 secreted by HT 1080 cells appear at ~92 kDa. [20]

Murine albumin measurement. A murine albumin ELISA quantitation kit (Bethyl Laboratories, Inc., Montgomery, TX, USA) was used to measure albumin in BALF [21] following the manufacturer's protocol. Briefly, Nunc Maxisorp plates (VWR International, Ltd.) were coated with 10 μg/ml capture antibody (goat anti-mouse albumin) for 1 h in bicarbonate buffer, followed by blocking with 1% BSA for 30 minutes. Samples and standard curve samples for mouse albumin were added to the plates after a washing step and left to incubate for 1 h. Plates were washed and goat anti-mouse albumin-HRP conjugate was added and incubated for 1 h. TMB substrate was

used to reveal positive reactions as previously described in the MPO assay. This assay was performed at room temperature.

Statistical analysis. Data were analyzed by Student's *t*-test and one-way ANOVA and illustrated in figures as mean ± SEM.

Results

Total cell counts in BALF were reduced in rac2$^{-/-}$ mice following immune complex-mediated ALI. To determine the effects of the *rac2* gene deletion on airway inflammation in the IC-mediated model of acute lung injury, WT and *rac2*$^{-/-}$ mice were treated by airway injection of anti-ovalbumin and intravenous injection of a 25-fold excess of ovalbumin. This procedure results in the deposition of IC in the airways of animals, [22] leading to profound tissue edema, inflammation, and lung injury within 6 h of treatment. As expected, we found that total cell counts in the airways of WT mice treated with IC increased in response to IC deposition in the airways and lung parenchyma (Figure 6.1a) which serves as a positive control and confirms IC deposition indirectly in the lung of both WT and *rac2*$^{-/-}$ mice. However, *rac2*$^{-/-}$ mice did not show significant increases in cell numbers retrieved from BALF (Figure 6.1a). Furthermore, differential staining of BALF cells revealed a predominance of neutrophils in WT but not *rac2*$^{-/-}$ mice, showing alveolar macrophage as the dominant cell type in BALF samples from *rac2*$^{-/-}$ mice (Figure 6.1b).

Rac2$^{-/-}$ *animals were protected from tissue destruction.* Lung histology was carried out to determine the presence of injury in our model. Following hematoxylin and eosin (H&E) staining of unlavaged control and IC-treated WT and *rac2*$^{-/-}$ mouse lung (Figure 6.2), WT mice showed loss of alveolar architecture and massive hemorrhage in large areas of the lungs, while in other regions, severe congestion and mucosal edema were evident. Both regions showed prominent neutrophil infiltration that reached the alveolar space. The lung histology seen in *rac2*$^{-/-}$ animals exposed to IC was not significantly different from control mice, where a slight congestion and neutrophil

infiltration were observed, in addition to moderate mucosal edema in the airways. Moreover, there was no hemorrhage or loss of lung architecture in *rac2*$^{-/-}$ in comparison to WT mice, suggesting that lung injury was absent in *rac2*$^{-/-}$ mice (Figure 6.2).

Endothelial destruction is absent and vascular permeability is maintained in rac2$^{-/-}$ *mice with ALI.* Vascular permeability is compromised when damage occurs in the vascular endothelial lining. This damage results from the release of granule-derived mediators and ROS during activation of inflammatory cells. We have shown previously that primary granule MPO and NE release is inhibited in *rac2*$^{-/-}$ mice (Figure 4.1c), and Rac2 is also required for optimal activation of ROS generation from neutrophils. [11] To determine the degree of loss of vascular integrity and increase in vascular permeability, we measured the levels of albumin in BALF samples from WT and *rac2*$^{-/-}$ mice with ALI. Albumin levels were increased in BALF from WT mice with ALI in comparison to controls, while in *rac2*$^{-/-}$ mice, changes in BALF albumin levels were negligible in comparison with control animals (Figure 6.3).

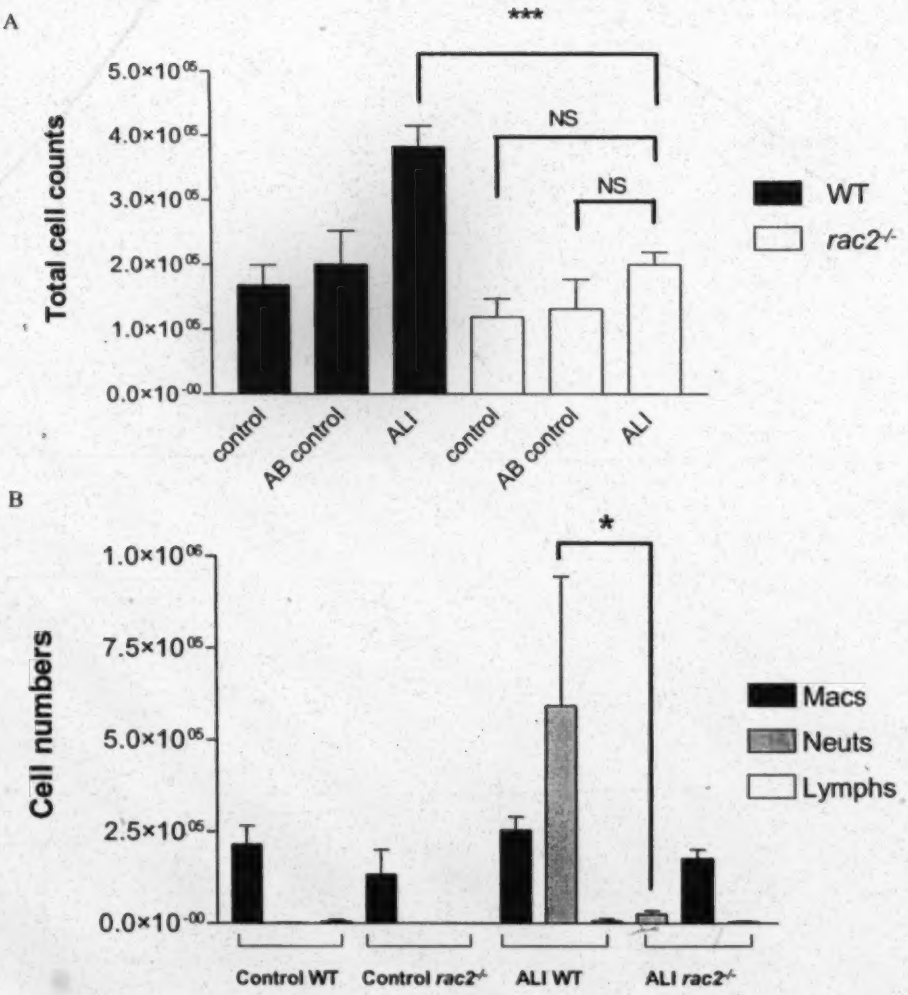

Figure 6.1. Total and differential BALF cell counts.
A) BALF total cell counts done by hemocytometer n = *7-8* for control experiments, n = *3-4* for antibody (AB) controls, and n =*11-12* for ALI . AB controls received an intratracheal injection of 16 µg/mouse of rabbit anti-ovalbumin IgG and sacrificed after 6 hours, NS = not significant, statistical significance was measured by Student's t- test. B) Numbers of neutrophils, macrophages, and lymphocytes in BALF as assessed by Diff-Quik staining. n = 3-4 for controls, and n = 8-9 for ALI, statistical significance was measured by ANOVA. *** $p < 0.001$, * $p < 0.05$. (Figure 6.1 was generated by D. Abdel Latif)

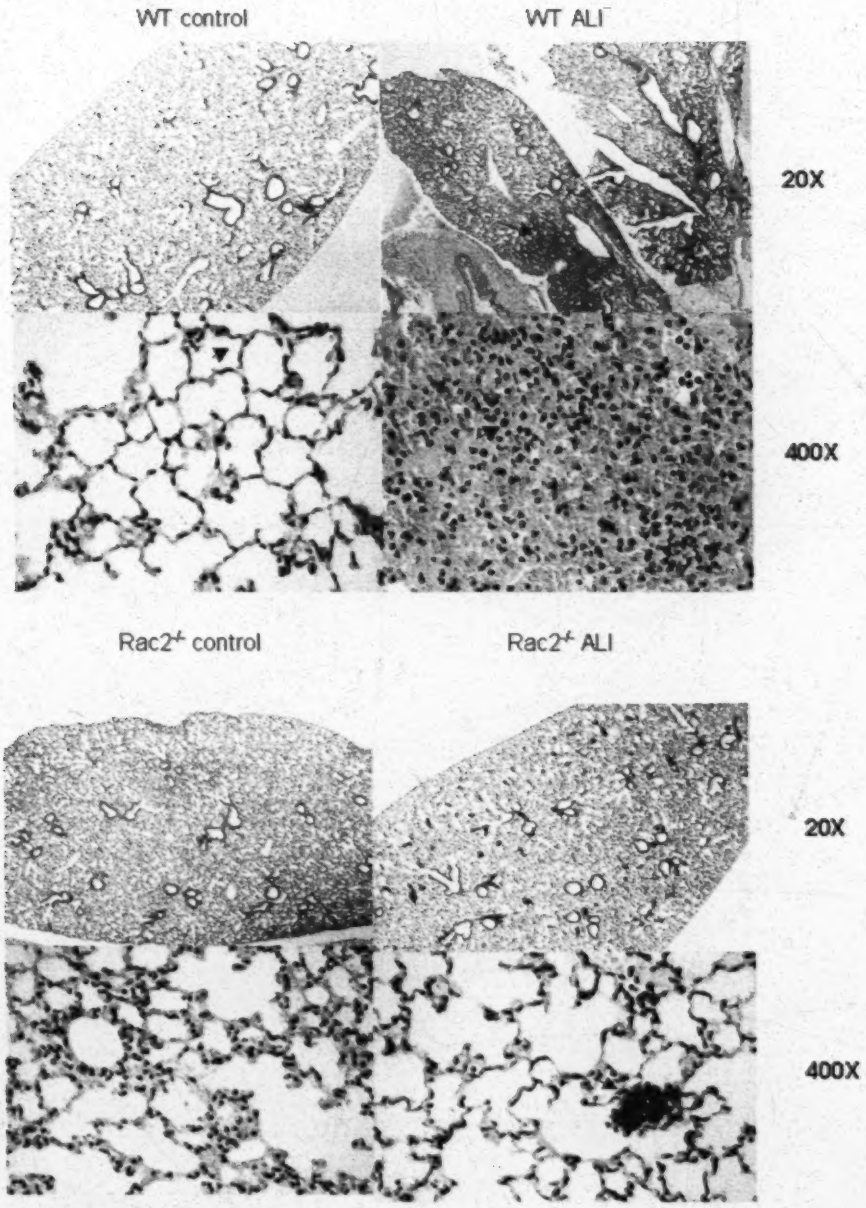

Figure 6.2. Lung histology of control and ALI.
Lungs were fixed with formalin and stained with H&E. Arrows show neutrophils in lung parenchyma and alveolar spaces. 20X and 400 X indicate the magnification. Arrows point at areas of hemorrhage and necrosis, and extravasated neutrophils in WT and *rac2*$^{-/-}$ ALI respectively. (Figure 6.2 was generated by D. Abdel Latif and L. Puttagunta)

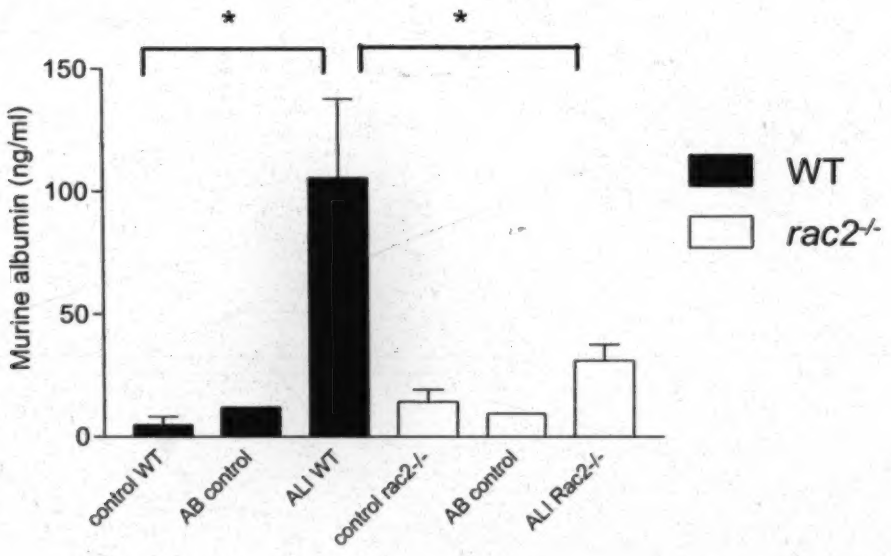

Figure 6.3. Reduced vascular permeability in *rac2*$^{-/-}$ mice with ALI.
Levels of mouse albumin in control and ALI BALF samples from WT and *rac2*$^{-/-}$ mice were determined by murine albumin ELISA. $n = 3\text{-}4$ for controls, $n = 2$ for AB controls, and $n = 7\text{-}8$ for ALI, * $p < 0.05$. (Figure 6.3 was generated by D. Abdel Latif)

Neutrophil mediator release is altered in rac2$^{-/-}$ mice. Our earlier *in vitro* observations showed that neutrophils from *rac2*$^{-/-}$ mice were unable to secrete MPO but released LTF from secondary granules and MMP-9 from tertiary granules in response to chemoattractant stimulation. [12] We therefore tested the possibility that differential release of neutrophil granule products also occurred *in vivo* during ALI. BALF samples were collected from WT and *rac2*$^{-/-}$ mice with ALI and tested for MPO, LTF, and MMP-9 activity. While MPO activity was detected in BALF samples from WT mice with ALI, MPO release was reduced in BALF collected from *rac2*$^{-/-}$ mice (Figure 6.4). In addition, the levels of LTF in BALF from WT mice with ALI were elevated over values from control mice (Figure 6.5a). However, LTF levels were similar in *rac2*$^{-/-}$ mice and WT mice with ALI. Unexpectedly, LTF levels were also increased in Ab controls. This finding suggests that LTF release from neutrophils and lung epithelium was maintained in *rac2*$^{-/-}$ mice exposed to the IC deposition in the lungs. MMP-9 activity, as assessed by gelatin zymography, was decreased in BALF samples from *rac2*$^{-/-}$ in comparison to WT mice (Figure 6.5b). Samples on the zymography gels were run separately, however, consistent experimental practice, through running the samples on the same day, and using the same incubation time and solutions, was employed to compensate for not testing the control and ALI samples on the same zymography gel.

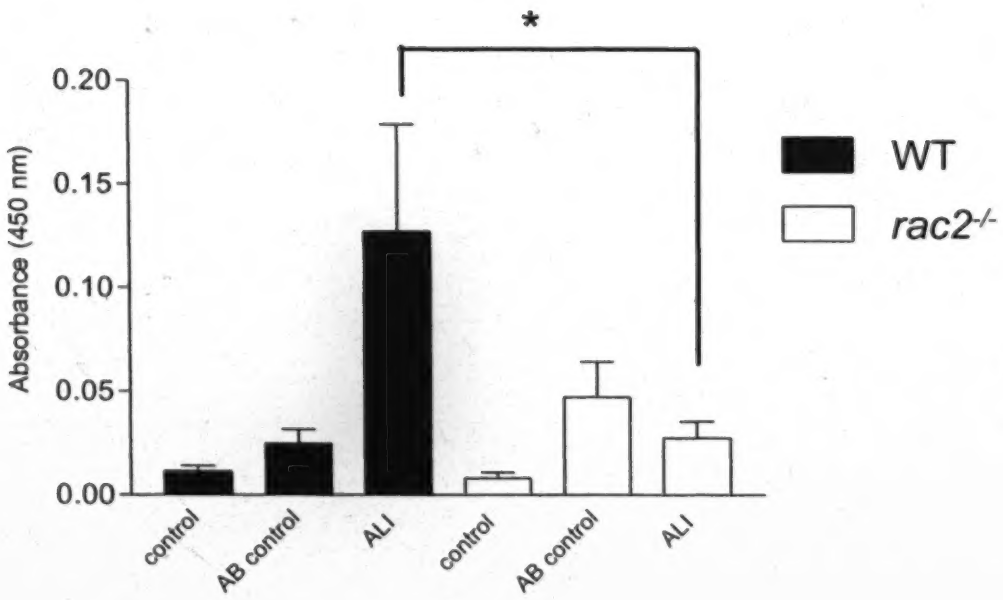

Figure 6.4. Decreased MPO activity in *rac2*$^{-/-}$ mice with ALI.
Levels of MPO activity in control and ALI BALF samples from WT and *rac2*$^{-/-}$ mice were assessed by colorimetric assay. Values are presented as absorbance measurements at 450 nm wavelengths. $n = 8\text{-}9$ for controls, $n = 5\text{-}6$ for AB controls, and $n = 11\text{-}13$ for ALI, * $p < 0.05$. (Figure 6.4 was generated by D. Abdel Latif)

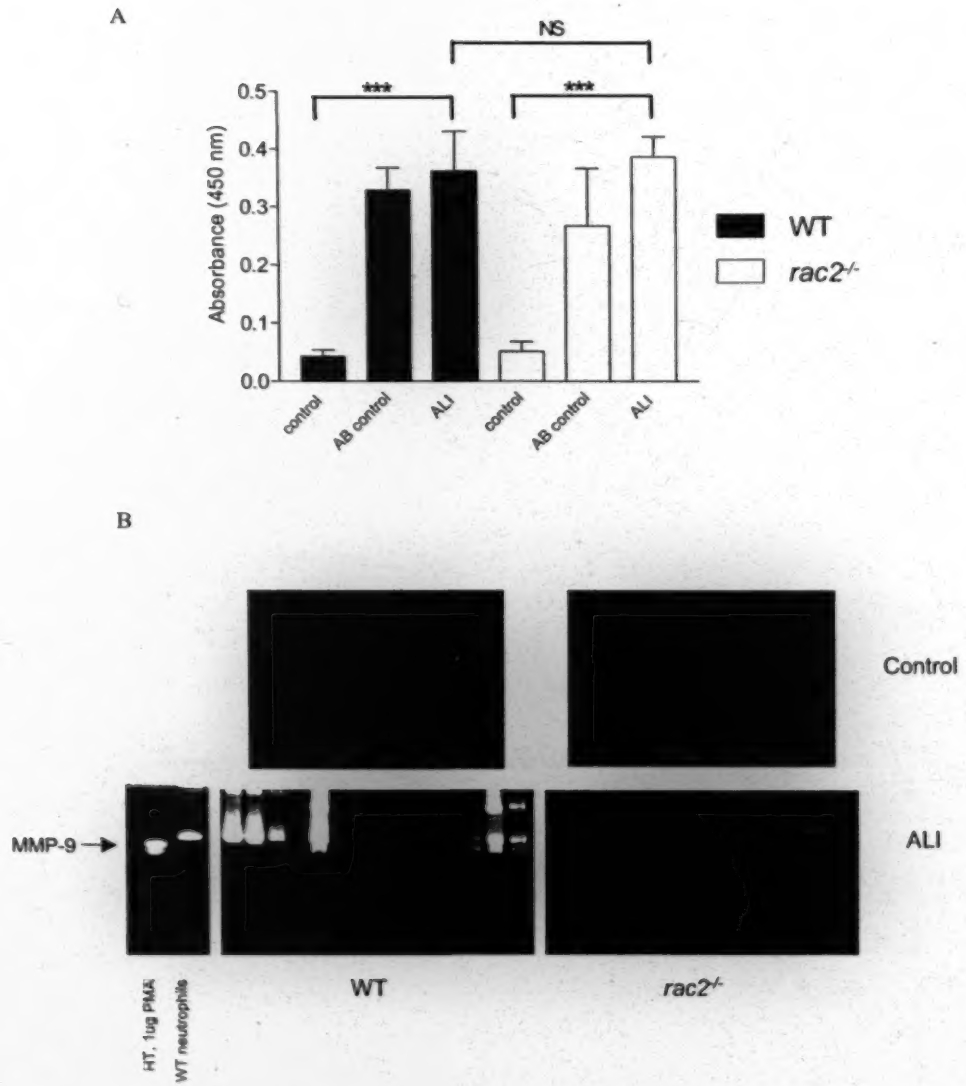

Figure 6.5. Secondary and tertiary granule products in BALF.
A) Secondary granule release as assessed by LTF ELISA. *n = 7-8* for controls, *n = 5-6* for AB controls, and *n =9-13* for ALI. B) Zymography of BALF from control WT and *rac2*$^{-/-}$ mice in comparison to mice with ALI. HT 1080 cells stimulated with 1 µg PMA were used as a positive control. Ten µl of BALF were mixed with 2.5 µl of zymography loading buffer in each well. *n = 7-8* for controls and *n=11-14* for ALI. WT and *rac2*$^{-/-}$ samples were run in separate gels and received the same incubation intervals simultaneously in the same wash/incubation/stain/destain solutions. (Figure 6.5 was generated by D. Abdel Latif)

Discussion

The findings in this study suggest that Rac2 regulates neutrophil functions in IC-mediated inflammation. Rac2 deficiency inhibits mediator release from primary granules in peritoneal and bone marrow neutrophils. [12] Roberts *et al.* were able to show that loss of Rac2 gene expression resulted in diminished ROS production. [11] The effects of Rac2 gene deletion on neutrophil functions have not previously been examined in the context of an acute inflammatory model. In the present study, we utilized an *in vivo* model of neutrophil-mediated injury by the use of an IC-mediated model of ALI. We demonstrated that *rac2*$^{-/-}$ knockout led to significantly reduced inflammation and tissue injury in the lungs of mice with ALI. This was assessed by established lung injury parameters such as total cell counts in BALF, neutrophil influx in lung parenchyma, loss of vascular integrity, and hemorrhagic edema in histology sections. [23,24] Neutrophil influx was apparent in lung tissue sections, particularly in regions associated with the epithelial layers surrounding the bronchi, but no significant increases in neutrophil numbers were observed in the airways of *rac2*$^{-/-}$ mice with ALI as determined by differential cell counts in BALF samples. Furthermore, there was a loss of recognizable tissue structure in lung section of wild type mice exposed to IC, and the lack of this feature in *rac2*$^{-/-}$ mice indicated that there is a significant discrepancy in reactions towards injurious stimuli. This discrepancy may preserve tissue structure and function as seen in the knockout mice. Hence, the absence of Rac2 gene expression is proposed to be protective against lung tissue destruction in this model.

The inflammatory cascade in IC-mediated ALI is initiated by cross-linking of FcγRIII receptors on macrophages that reside normally in the airways [25]. This leads to the

production of cytokines and chemokines that activate epithelial cells to produce complement 5a as well as inflammatory cytokines (e.g. TNF, IL-8, MIP-1α, MIP-1 β, RANTES, and MCP-1). These cytokines have an important role in propagation of the inflammatory response through the upregulation of adhesion molecules on vascular endothelium and facilitation of neutrophil extravasation. Neutrophils circulating in the bloodstream become adherent to endothelial cells and transmigrate into the submucosal layers towards epithelial cells and the airways. Once neutrophils reach the airways, they become activated and release ROS and granule-derived mediators, which have been shown to induce edema, mucus production, hemorrhage and tissue injury in this model. [26,27] We are currently investigating the role of cytokines and chemokines (including TNF, MIP-1α, and MIP-1 β) in this model of acute lung injury in *rac2*$^{-/-}$ mice.

Macrophages also express Rac2, and Rac2$^{-/-}$ macrophages were shown to have a selective defect in phagocytosis of opsonized particles and O_2^- production. [28] This may contribute to the loss of lung injury in *rac2*$^{-/-}$ mice, particularly since this model is dependent upon FcγRc-mediated activation of macrophages. It is not yet established whether macrophage cytokine and chemokine release or degranulation responses are affected in *rac2*$^{-/-}$ mice or if macrophages are properly stimulated in the *rac2*$^{-/-}$ ALI by the deposition of OVA/anti-OVA immune complex. Thus, the role of macrophage in the IC-mediated ALI requires further investigation especially in the *rac2*$^{-/-}$ mice. [11]

The function of Rac2 in neutrophils is associated with a range of activities including chemotaxis, ROS production, and degranulation of primary granules. [12] Based on the findings reported here, it is possible that the loss of neutrophil migration to the airways is responsible for protection against tissue destruction in *rac2*$^{-/-}$ mice. Other

possible causes of diminished lung injury may be reduced ROS production and diminished release of primary granule products. [11]

Our previous findings demonstrated that *in vitro*-stimulated Rac2$^{-/-}$ neutrophils released substantially reduced levels of primary granule products but continued to release secondary and tertiary granule products. [12] Our present findings indicate that *in vivo* release of neutrophil MPO was also diminished, as we found that BALF levels of primary granule MPO were significantly reduced in *rac2*$^{-/-}$ mice with ALI compared with WT mice. The granule products, LTF and MMP-9, are secreted from neutrophils as well as other cell types such as bronchial epithelial cells. [29-32] However, Rac2 is not expressed outside cells belonging to the hematopoietic lineage. [13] Thus, Rac2 deficiency is unlikely to affect epithelial cell production of granule products. Our observation that LTF levels were elevated in BALF from *rac2*$^{-/-}$ mice with ALI supports our earlier finding that bone marrow neutrophils from *rac2*$^{-/-}$ mice secreted LTF at equivalent levels to WT cells. LTF is also secreted from lung epithelium and this process is not likely to be affected by the Rac2 deficiency, which indicated that its role in inducing tissue destruction is minor. However, MMP-9 was significantly decreased in BALF of Rac$^{-/-}$ ALI, which may be explained by a lack of neutrophil extravasation into the airways, diminishing the contribution of activated neutrophils to total airway MMP-9, and potentially adding to the protective effects of Rac2 gene deletion against tissue injury. [33,34] Since many different cell types produce MMP-9 (e.g., epithelial cells, macrophages, endothelial cells, and neutrophils), it is not possible to determine the tissue source of MMP-9 using the available assays. In conclusion, Rac2 deficiency contributes to a diverse cascade of events that affect inflammatory cell functions, leading to attenuation of IC-mediated lung

injury. Rac2 inhibition is one strategy to consider in future pharmacological approaches against neutrophil-mediated inflammation due to its potential role in preventing tissue destruction and loss of organ function.

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Chapter 7

7.1 Discussion

The results of this research project have shed some light on the role of Rac2 in neutrophil functions and in neutrophil-mediated inflammatory responses. Neutrophils act as a double-edged sword, where optimal activation is crucial to the innate immune system, while inadequate or excessive activation may jeopardize the life of a human being.

Results from the study of superoxide release in granulocytes revealed diverse mechanisms of neutrophils and eosinophils in activating NADPH oxidase. The discrepancy seen in extracellular superoxide production between eosinophils and neutrophils was due to preferential intracellular assembly of NADPH oxidase subunits in neutrophils during superoxide production. Conversely, eosinophils were shown to produce ROS extracellularly. This discrepancy may be explained by the different roles these cells play in the immune response. In asthma, however, extracellular release of superoxide may lead to tissue damage and contribute to asthma pathogenesis. Hence, anti-oxidant pharmacotherapy may be beneficial if eosinophilia occurs in the lung or bloodstream of patients with unstable asthma.

A. phagocytophilum infection of neutrophils resulted in intracellular assembly of NADPH oxidase in vacuoles containing bacteria. It was postulated that a failure in the

NADPH oxidase assembly and activation occurred during neutrophil infection by *A. phagocytophilum*. However, we were able to show that the ability to produce ROS in infected neutrophils was intact. Since ROS are cytotoxic to *A. phagocytophilum*, this suggested that ROS were somehow neutralized by the bacterium upon interaction with the neutrophil during infection. Furthermore, *A. phagocytophilum* were shown to reside in oxidase-free vacuoles that lacked membrane components of the NADPH oxidase system as a protective step against the bactericidal effect of the oxidase. This study provided a better understanding of mechanisms of host evasion by *A. phagocytophilum*.

The role of Rac2 in neutrophil degranulation was investigated in this thesis. Rac2 has already been well investigated for its function in superoxide production and actin cytoskeleton dynamics. A novel role for Rac2 in the selective release of primary granules was determined. Chemoattractant stimulation was shown to utilize a common pathway including Rac2 and actin cytoskeleton, leading to granule mobilization and release. Other populations of granules were exocytosed normally in $Rac2^{-/-}$ neutrophils, indicating different pathways mediating their release. Furthermore, superoxide production was diminished in $Rac2^{-/-}$ neutrophils in spite of normal or increased expression of other Rho GTPases. Common signaling pathways were also shown to be utilized for both degranulation and superoxide generation downstream of chemoattractant stimulation. Expression of other isoforms of Rho GTPases could not compensate for the Rac2 deficiency, which had a detrimental effect on these two biological functions. In conclusion, Rac2 was found to have an essential role in mediating immune functions in neutrophils, which strongly implicates it as a target for anti-inflammatory therapy in inflammatory disorders.

Finally, to explore the biological significance of Rac2 in neutrophil activation, we tested the effect of Rac2 deficiency in a lung injury model. Lung tissue injury was substantially diminished in $Rac2^{-/-}$ mice as assessed by inflammatory parameters. These findings indicate that Rac2 deficiency alters a diverse cascade of events that affect inflammatory cell functions, leading to attenuation of lung injury. Hence, Rac2 inhibition may be considered for future pharmacological approaches against neutrophil-mediated inflammation due to its potent effect in preventing tissue destruction and loss of organ function.

7.2 Pattern of reactive oxygen species (ROS) production in neutrophils and eosinophils

ROS production is an important bactericidal process that starts with the generation of $O_2^{\cdot-}$. The NADPH oxidase system is responsible for the production of $O_2^{\cdot-}$ from granulocytes upon stimulation. In this project, two types of granulocytes were investigated, eosinophils and neutrophils, and their production of ROS in normal and asthmatic subjects.

Neutrophils have been shown to be involved in the pathogenesis of bronchial asthma [1-3]. However, their influx in the airways is associated with certain non-allergic stimuli [4-6], and is usually transient, although neutrophils are the first cell to transmigrate to the lung parenchyma following endobronchial allergen challenge [7-9]. Neutrophils have been shown to play a dangerous and sometimes fatal role during asthma attacks and exacerbations, where the severity of nocturnal asthma is directly proportional to the number of neutrophils in the lungs [10] and the presence of neutrophils indicates a poor outcome and corticosteroid resistance [11]. The hallmark cell in acute exacerbation of asthma is the neutrophil, with or without infection [3,6,12].

It has been previously reported that the NADPH oxidase activity in a neutrophil is 10 times less than that of the eosinophil. This was explained by either a higher expression of NADPH oxidase in eosinophils, or an inability to measure released $O_2^{\cdot-}$ from activated neutrophils due to its intracellular site of production [13-19]. However, the sites of NADPH oxidase assembly in eosinophils are largely unknown. Hence, in this project, I aimed to determine the mechanisms by which the discrepancy between neutrophils and eosinophils in levels of extracellularly released $O_2^{\cdot-}$ occurs. Furthermore, subcellular localization of

the NADPH assembly indicates where O_2^- is released in each cell type. To reach these goals, confocal imaging was employed due to its ability to discriminate subcellular components by fluorescent labeling of fixed cells.

Being an important subunit of the NADPH oxidase system, Rac2 was used as a cytosolic marker for NADPH activation and assembly in both neutrophils and eosinophils. The $p22^{phox}$ subunit was also used as a membrane marker for NADPH oxidase. In resting cells, Rac2 was mainly localized in the cytosol in both neutrophils and eosinophils, with no apparent granular distribution. Membrane-bound $p22^{phox}$ immunofluorescence showed distribution around the plasma membrane. Some intracellular localization of $p22^{phox}$ was seen, however, its exact subcellular compartment could not be determined. Upon NADPH activation, Rac translocates to the plasma membrane where it binds to $p67^{phox}$, initiating the process of O_2^- production [20,21]. Rac2 translocation was evident in both neutrophils and eosinophils stimulated with PMA. However, the destinations of Rac2 were different in each cell type, where Rac2 translocated mainly to the cell membrane in eosinophils and showed colocalization around the periphery of the cells with $p22^{phox}$. Conversely, a clear intracellular and possibly vacuolar colocalization of Rac2 and $p22^{phox}$ was observed in neutrophils. Levels of Rac2 and $p22^{phox}$ expression were similar between neutrophils and eosinophils in captured images during rest and activation, indicating similar expression levels in both cell type which was consistent with the PCR data generated using human neutrophils and eosinophils (Figure 2.3). Intracellular ROS production was determined by using dihydrorhodamine-123, a fluorescent probe used to detect intracellular and extracellular

ROS production [22], which showed equivalent levels of ROS production in neutrophils and eosinophils.

In conclusion, neutrophils preferentially generate intracellular O_2^-, while eosinophils tend to generate O_2^- at the cell membrane with subsequent extracellular release of the superoxide anion. This pattern fits well with the proposed immune function for neutrophils, in which they function as professional phagocytes with intracellular bacterial killing abilities. Eosinophils, on the other hand, are comparatively non-phagocytic cells with an anti-tumor [23] and anti-parasitic [24,25] role, in which intracellular killing is not required. That does not deny though their ability to engulf bacteria, but suggests that they do not possess the level of machinery for efficient killing that neutrophils and macrophages have [26-30].

7.3 *Mechanism of host evasion by* Anaplasma phagocytophilum *in neutrophils*

A. phagocytophilum causes human granulocytic anaplasmosis (HGA), which is a tick-borne disease. Following tick bite, the bacterium is engulfed by activated neutrophils in the tissue. However, this process of receptor-mediated endocytosis [31] does not end with killing of *A. phagocytophilum*, but rather leads to the infection of neutrophil itself where the bacteria flourish and cell-to-cell infection occurs. As a result, host defense mechanisms lead to the release of cytokines and evolution of an inflammatory response producing symptoms [32]. Symptoms are typically symptoms of acute inflammation where fever, malaise, body aches and headache are experienced by the patient. Laboratory test results reveal leukopenia, thrombocytopenia and high levels of C-reactive protein and liver enzymes. HGA tends to be self-limiting in most cases, yet severe inflammatory

responses may occur in some instances and lead to fatal consequences (e.g. acute renal failure and shock) [33]. Proliferation of *A. phagocytophilum* in neutrophils indicates the ability of the bacterium to escape the killing machinery inside neutrophils. In earlier studies, it was suggested that *A. phagocytophilum* evades killing through disruption of the bactericidal ability of neutrophils through the inhibition of phagocytosis [34] or NADPH oxidase activation [35-37]. In HL-60 cells, Rac2 and gp91^{phox} expression was diminished due to *A. phagocytophilum* infection [35,36]. As a result, ROS production was diminished and the bacteria survived inside neutrophils. Human neutrophils are terminally differentiated cells that express Rac2 and gp91^{phox} before reaching the site of infection [38]. As superoxide production is jeopardized in infected cells, this suggested that *A. phagocytophilum* employs an alternative mechanism to inhibit ROS production in neutrophils. Unpublished observations indicate that *A. phagocytophilum* may act to evade initial killing following infection of neutrophils by expression of cytochrome oxidase on its cell surface, which inactivates ROS (Jason Carlyon, personal communication).

Colocalization of Rac and p22^{phox} was used to indicate the assembly and close proximity of cytosolic and membrane components of NADPH oxidase upon stimulation of respiratory burst. To induce respiratory burst, PMA was used as the stimulus in normal and infected cells. Control neutrophils showed a typical distribution of diffuse cytoplasmic Rac staining which did not colocalize with the plasma membrane and intracellular aggregations of p22^{phox}. Colocalization of Rac and p22^{phox} was evident in PMA-stimulated cells, which was consistent with the assembly of NADPH oxidase [39,40]. NADPH oxidase assembly was observed in infected neutrophils, as determined by intense colocalization of Rac and p22^{phox} immunoreactivities, indicating that neutrophil

ability to produce intracellular O_2^- was preserved. A synergistic effect was seen upon PMA stimulation of infected neutrophils, in which the plasma membrane was the main site of Rac/ $p22^{phox}$ colocalization, which may be due to the effects of PMA preferentially driving the assembly of NADPH oxidase at the cell membrane [41]. These observations were further confirmed by flow cytometric data where functional cytochrome b_{558} was shown to be assembled on the cell membrane in infected neutrophils, which indicates NADPH activation.

Hence, *A. phagocytophilum* does not utilize an inhibitory mechanism against NADPH oxidase assembly and activation during acute infection in neutrophils. Neutralizing intracellularly released ROS by cytochrome oxidase activity may be the mechanism by which *A. phagocytophilum* escapes killing upon engulfment. Another mechanism that was proposed in this study was that membranous components ($gp91^{phox}$ and $p22^{phox}$) were expressed on granular membrane but not on *A. phagocytophilum*-containing vacuoles in infected neutrophils, suggesting that the formation of a protective isolation vacuole facilitates *A. phagocytophilum* survival in neutrophils.

7.4 Mechanisms regulating neutrophil degranulation in response to chemoattractant stimulation

Degranulation is a central function of activated neutrophils. Initially, it was thought that degranulation was only essential for intra-phagosomal bacterial killing. However, recruitment of other inflammatory cells involved in the innate and adaptive immune response was shown to occur as a result of extracellular release of granule proteins such as defensins [42-44]. Neutrophil migration is facilitated by tertiary granule contents (gelatinase B) through their role in degrading matrix proteins during

extravasation [45]. Having established a role for Rac2 in vesicle trafficking and exocytosis from mast cells, this regulatory protein was a strong candidate for mediating neutrophil degranulation [46-48]. The area of granule mobilization, docking and release is largely open for mechanistic studies to address the pathways governing exocytosis of different granule populations.

In our study, we used the same *rac2*$^{-/-}$ model generated in the earlier study by Roberts *et al.* Upon chemoattractant stimulation of neutrophils with f-Met-Leu-Phe and LTB_4, we assayed for the extracellular release of granule products (Table 7.1) [49,50]. MPO [51,52] and NE [53] were assayed as marker enzymes for primary (azurophilic) granule release. The activation of MPO and NE release was abolished upon f-Met-Leu-Phe and LTB_4 activation in Rac2$^{-/-}$ neutrophils. This suggested that Rac2 selectively regulates the release of primary granules from neutrophils stimulated through GPCRs. Interestingly; these findings also indicate that Rac1 could not substitute for Rac2 in the degranulation pathway. The granule contents of *rac2*$^{-/-}$ and wild type neutrophils were assessed by electron microscopy and flow cytometry analysis to detect any possible deficiencies in granule formation. Primary granule numbers and contents were equivalent in wild type and *rac2*$^{-/-}$ mice. Hence, granulogenesis was intact in Rac2$^{-/-}$ neutrophils, and the defect in granule exocytosis was attributed to the deficiency in Rac2 expression. This in turn suggests that Rac2 is the only Rac isoform involved in activation of degranulation of primary granules by f-Met-Leu-Phe and LTB_4 receptors in neutrophils. Furthermore, a surface marker for primary granule, CD63 [54], was used to assess for granule mobilization in activated cells, in an attempt to further explore the signaling mechanism downstream of Rac2 during degranulation. Failure of granule mobilization to submembranous area,

which is the initial step of degranulation, was noted in *rac2*$^{-/-}$ cells upon f-Met-Leu-Phe stimulation. This is in agreement with previously published studies that highlighted the role of Rac2 in actin cytoskeleton remodeling and the formation of filamentous actin [55,56] an essential step for granule mobilization [57].

To assess if primary granule secretion may involve other Rho GTPases, we utilized an established priming agent for neutrophils (TNF). TNF was used previously in Rac2$^{-/-}$ neutrophils to prime cells for superoxide production, and it led to a correction of defective $O_2^{\cdot-}$ production [58]. In our studies, *in vitro* priming with TNF did not rescue the degranulation defect seen in f-Met-Leu-Phe-stimulated *rac2*$^{-/-}$ bone marrow neutrophils. *In vivo*-primed cells were also used to assess any correction of the degranulation defect. Cells were obtained from peritoneal exudate (PE) of *rac2*$^{-/-}$ mice and assayed for MPO release. Once again, *rac2*$^{-/-}$ PE neutrophils failed to release a significant amount of MPO in comparison with resting values seen in wild type and *rac2*$^{-/-}$ bone marrow neutrophils.

Secondary and tertiary granule exocytosis was measured by LTF [59,60] and MMP-9 [61,62] respectively. LTF release was preserved in Rac2$^{-/-}$ neutrophils, as seen by LTF ELISA, in response to PMA and f-Met-Leu-Phe in adherent cells and cells in suspension. MMP-9 measured by gelatin zymography showed that Rac2$^{-/-}$ neutrophils release their tertiary granule contents in levels comparable to wild type cells.

The kinase p38 MAPK is a signaling molecule that is classically activated in response to TNF stimulation [63-65]. It was shown in numerous studies that p38 MAPK is involved in vast array of neutrophil functions, including cell motility, actin reorganization and receptor-mediated signaling [66-70]. Due to the profound defect in granule mobilization and exocytosis observed in Rac2$^{-/-}$ neutrophils, a further investigation of a potential

signaling link between Rac2 and p38 MAPK in primary granule release pathway was carried out. An assessment of p38 MAPK activation in response to f-Met-Leu-Phe and TNF was performed using an anti-phospho-p38 antibody and the resulting bands were compared to the total cell p38. $Rac2^{-/-}$ neutrophils showed normal phosphorylation of p38 MAPK, which indicated that Rac2 might be downstream of p38 in the formyl and TNF receptor-signaling pathway in murine neutrophils. Especially the p38 activation was shown to occur as a response to fMLP stimulation of neutrophils and this activation leads to the release of elastase from primary granules which was blocked by SB203580 a specific inhibitor of p38 MAPK [71]. In summary, an involvement of Rac2 in chemoattractant-induced degranulation from murine neutrophils was established in this study. This was a selective mechanism that was highly dependent on Rac2 and could not be corrected by priming agents either *in vivo* or *in vitro*. Furthermore, Rho GTPases that are also expressed in neutrophils and have been shown to substitute for Rac2 in different neutrophil functions [58,72], failed to substitute for primary granule release. Hence, Rac2 is a candidate for further studies aimed at expanding our knowledge of downstream effector molecules mediating actin cytoskeletal changes leading to granule mobilization and subsequent release upon chemoattractant stimulation in murine and human neutrophils.

7.5 Rac2 is central in neutrophil mediated signaling

Rac2 GTPase is the main Rac isoform expressed in human neutrophils [73] and is equally expressed with Rac1 in mouse neutrophils [74]. Rac1 and Rac2 display a high degree of homology. However they have distinct roles in mediating effector functions in human and mouse neutrophils. Rac2 was shown to be the main isoform regulating main steps of chemotaxis starting from the interaction with activated endothelium to intracellular bacterial killing mechanisms especially superoxide production and degranulation in rodents [58,75-77].

Based on our previous study of Rac2 signaling in neutrophils [78], a common signaling pathway between primary granule release and $O_2^{\cdot-}$ production was proposed. On one hand, a profound defect was observed in chemoattractant-mediated primary granule mobilization and release that could not be corrected by priming of neutrophils. On the other hand, $O_2^{\cdot-}$ production did not reach its maximal level in *rac2*$^{-/-}$ neutrophil when compared to wild type cells. This pathway appears to have a divergence point downstream of Rac2 due to the fact that PMA induces superoxide production from neutrophils by activation of the NADPH oxidase complex through PKC stimulation [79]. However, PMA did not evoke primary granule release from murine bone marrow neutrophils, although it had a stimulatory effect on secondary granule release in both WT and Rac2$^{-/-}$ cells. The diacylglycerol (DAG)-sensitive C2 domain of PKC serves as a target for phorbol ester stimulation, and proteins containing this domain include conventional isoforms of PKC [80]. Recently, Rac was shown to function upstream of PKC during secretory granule release in RBL-2H3 cells [81]. Hence, PKC may represent a split

in signaling pathways downstream of Rac2 which have not yet been identified for primary granule exocytosis (Figure 7.1).

These findings suggest that intracellular targets of PMA differentially activate specific pathways of mediator release from neutrophils. Intracellular PMA targets are likely to include conventional PKC isoforms, as these have been shown to be involved in the induction of $O_2^{\cdot-}$ production from neutrophils [82]. The lack of effect of PMA on primary granule exocytosis suggests that PKC-mediated phosphorylation of intracellular effector molecules is not involved in this process; however, PKC is likely to be involved in secondary and tertiary granule release.

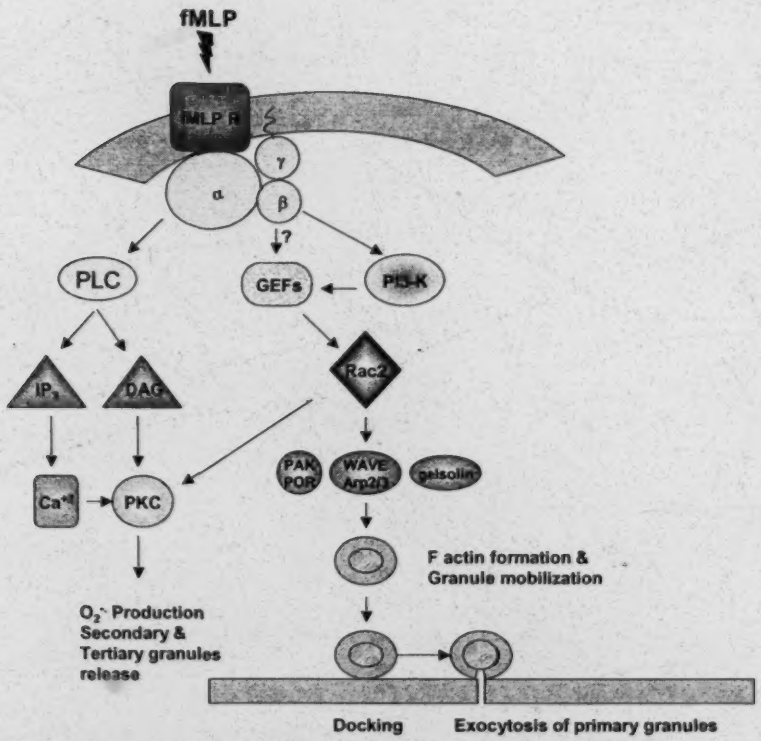

Figure 7.1. Proposed signaling pathway mediating superoxide production and degranulation in neutrophils upon f-Met-Leu-Phe stimulation.
According to the data obtained in this study, a common pathway is suggested for superoxide production and degranulation, where Rac2 is a point of divergence. Effectors utilized for superoxide production include PKC isoforms, while PAK, POR, WAVE, Arp2/3, and gelsolin are proposed to mediate Rac2 effects on the actin cytoskeleton leading to granule mobilization and exocytosis.

7.6 Neutrophils in lung diseases: friend or foe?

Neutrophils are a key cell population in the innate immune system, performing microbial killing, activation in other innate immune cells, and propagation of the inflammatory response through chemotaxis, phagocytosis, degranulation, and the generation of reactive oxygen metabolites. Disturbances in neutrophil functions lead to immune deficiency, where an increased susceptibility to bacterial and fungal infection is observed [83-86]. Overexuberant neutrophil activation occurs when the regulatory mechanisms of neutrophil functions fail or are overwhelmed in severe sepsis or trauma. This, in turn, leads to neutrophil-mediated tissue injury that can prove fatal in many conditions such as ALI leading to multi-organ systemic failure.

Neutrophils have been shown to be involved in several respiratory disorders including chronic obstructive pulmonary diseases (COPD) [87], ALI [88,89] as well as its more severe form, ARDS [90] and asphyxic episodes of bronchial asthma [11,91,92]. In ALI, it is postulated that injury of either the lung endothelium or epithelium triggers an inflammatory cascade. However, systemic activation of inflammatory cells, as seen in sepsis or major burn injuries, also leads to lung damage.

Tissue damage occurring in the lung may be mediated by ROS production, through the assembly and activation of NADPH oxidase system and O_2^- production. MPO, a primary granule enzyme, potentates the effects of ROS production from activated neutrophils upon NADPH oxidase activation. Oxidative damage to the endothelium leads to the disruption of intercellular tight junctions and compromises vascular integrity [93]. Another neutrophil-derived mediator is NE, which has the capability to degrade surfactant proteins A and D, leading to prolonged lung inflammation [94-96]. Furthermore,

NE was shown to stimulate the release of inflammatory cytokines from lung epithelium and promotes apoptosis in these cells, an event that is pro-inflammatory in the lungs [97-100]. Other components of neutrophil granules (e.g. MMPs [101,102], defensins [103-105]) have also been shown to play a role in inducing tissue damage in ALI.

Altogether, this suggests that an imbalance in the regulatory mechanisms mediating the microbicidal functions of neutrophils is responsible for ALI. Hence, targeting specific molecules in order to avoid their injurious effect must be thoroughly studied due to the possibility of rendering the host susceptible to infection if blockade of neutrophil immune function takes place.

However, some debate still exists regarding the role of neutrophil in lung injury. ALI has been noted in neutropenic patients, which may imply a minor role for neutrophil over-activation in lung tissue destruction [106,107]. Nevertheless, the substantial amount of research conducted on lung injury model strongly indicates that targeting neutrophil products as NE and ROS may not prove beneficial in decreasing the morbidity and mortality of ALI patients, particularly if the function of regulatory proteins such as Rac2 is the main driving force behind the release of those mediators. Hence blocking Rac2 may be the next logic step to prevent the release of a range of toxic mediators in acute inflammatory events which are implicated in tissue destruction and cytotoxicity.

7.7 *Biological significance and clinical implication of targeting Rac2*

Many attempts have been made to inhibit or neutralize released neutrophil mediators [108] and oxidants [109], prevent neutrophil accumulation [110] and cytokine

inhibition [111]. All of these trials failed to demonstrate a significant effect in lowering mortality or morbidity in ALI patients.

It was established in this project, that Rac2 is a central molecule in mediating neutrophil functions *in vitro* and *in vivo*. Furthermore, the clinical implications of the results obtained were assessed using an *in vivo* model of ALI, which showed a beneficial role of Rac2 deficiency in preventing tissue destruction in the lung.

These data indicate that targeting Rac2 may prevent neutrophil-mediated inflammatory diseases through the inhibition of toxic mediator release and tissue destruction. This in turn may prove beneficial in preventing the loss of organ function seen in acute inflammatory disorders resulting from overexuberant neutrophil activation.

Rac2 inhibition may be achieved by various approaches (e.g. targeted gene disruption, antibody mediated inhibition). Targeted gene disruption seems remote in most human studies. Although shown to be beneficial in our ALI model in the mouse, Rac2 deficiency leads to an immune deficiency status in the *rac2* $^{-/-}$ mice with a higher predisposition to spontaneous infections in spite of breeding in a pathogen-free environment. Furthermore, a human dominant negative mutation of *rac2* led to a pathological status that required bone marrow transplantation to be corrected.

Antibody mediated inhibition of Rac2 protein may be achieved by the delivery of an anti-Rac2 antibody bound to a carrier molecule to allow its passage through the cell membrane. Different carrier proteins may be used, however, the delivery of a biologically active antibody to a certain intracellular compartment has not been described in human neutrophils. Further studies are required to elucidate a proper carrier for the Rac2-antibody capable of targeted intracellular delivery and a proper method of delivery

(inhalation vs intravenous injection).

7.8 Conclusions

An increasing body of evidence suggests that a major role for Rac2 in neutrophil function is emerging. Observations from other studies suggest that $O_2^{\cdot-}$ production, chemotaxis and cytoskeletal changes are dependent on Rac2. Our data suggest that a common signaling pathway exists between $O_2^{\cdot-}$ production and degranulation, where Rac2 plays a central and pivotal role in regulating the release of these products. We suggest also that a divergence of pathways occurs downstream of Rac2, where activation of different effectors may occur to activate both NADPH oxidase or granule mobilization and exocytosis. Rac2-mediated degranulation involved primary granule mobilization in activated neutrophils, and this was proposed to be dependent on actin cytoskeletal changes. Hence, inhibiting Rac2 may indeed impair mediator release from activated neutrophils. Such inhibition may have a beneficial effect in neutrophil-mediated inflammatory disorders that involve tissue injury and loss of organ function. Further studies are required to delineate the possibility of Rac2 inhibition *in vivo*, and the proper method of delivering the inhibitor to the host.

7.9 References

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